STUDIES ON THE EFFECT OF ATABRINE ON VITAMIN A METABOLISM

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(Received for publication August 14, 1951)

Many pathological conditions are known to affect adversely the metabolism of vitamin A in various ways, such as increasing utilization, hindering the conversion of carotene to vitamin A, or increasing the excretion of vitamin A by the kidneys. All these conditions tend to increase the vitamin A requirement of the body. Certain drugs also impair the metabolism of vitamin A and of carotene in one or more of these ways; e.g., liquid paraffin (Rowntree, '31; Dutcher, Harris, Hartzler and Guerrant, '34; Curtis and Ballmer, '39) and thiouracil (Cama and Goodwin, '49).

The antimalarial drug atabrine is administered prophylactically and in suppressive treatment over long periods of time. It has already been shown that this drug affects the metabolism of some important nutrients; e.g., calcium (Williamson, Hegsted, McKibbin and Stare, '46), thiamine (Hegsted, McKibbin and Stare, '45), choline (Hegsted, McKibbin and Stare, '44b), and vitamin B_{12} (Ershoff, '50). Because malaria is endemic in so many regions, additional knowledge relating to the effects of atabrine on essential nutrients is desirable. Experiments were, therefore, carried out with rats in order to study the effect of chronic atabrine administration on the metabolism of vitamin A.

METHODS

Rats weighing 35 to 50 gm were used. The food of the mothers consisted of sprouted wheat, barley, bran, skim milk pow-

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der and the seasonal vegetables. Two weeks before their progeny were weaned, vegetables were eliminated from their diet, thus reducing the carotene intake. The livers of the young after weaning contained 3 to 8 I.U. of vitamin A per liver. The young rats were then transferred to the experimental diet, which consisted of 50% skim milk powder, 43% starch, 5% olive oil, and 2% salt mixture. With this diet the liver was completely depleted of vitamin A within one week. In the atabrine-containing diet, 800 mg atabrine were incorporated per kilogram of ration. The rats of each experiment were pair-fed unless otherwise stated.

For determination of the vitamin A content of the liver and intestines, the rats were decapitated and the required organs removed. The intestines were flushed out with water and the washings were collected for analysis. The liver and the washed intestinal tract were saponified by boiling for 30 min. in alcoholic potassium hydroxide. The saponified mass was cooled, extracted with ether, and the ether extract dried over sodium sulfate. After evaporation of the ether chloroform was added to the residue and vitamin A determined by the Carr-Price reaction with the aid of a Klett-Summerson photoelectric colorimeter. Atabrine did not interfere with the determination of vitamin A when filter 66 was used. The intestinal contents were also extracted with ether and treated in the same way. Faeces were blended with water in a Waring Blendor. Carotene was extracted with petrol ether and determined colorimetrically.

RESULTS

Weight increase and food consumption

Three groups of young rats were fed the following diets for 6 weeks: group A, comprising 27 rats, received the experimental diet with atabrine ad libitum; group B, 27 animals, received the same diet without atabrine, but the amount given was restricted to the amount of food eaten by group A; group C, 6 rats, served as controls and were offered the ex-

perimental diet without atabrine ad libitum. All rats received a large supplement of vitamin A, as they were used for studies on vitamin A storage in the liver (see below).

This experiment showed that the inclusion of atabrine in the milk powder diet depressed the normal growth of young rats by decreasing their voluntary food intake. There was practically no difference in the weight increase between groups A and B (64 and 65 gm, respectively), but a very marked difference was noted between the weight increases of these groups and that of group C (101 gm). It seems that the bitter taste of diet A prevented the animals from consuming as much food as they would have eaten on the same diet without the drug. It follows from this experiment that atabrine incorporated in a milk powder-containing diet does not impair the utilization of food.

Storage of vitamin A

The purpose of the experiments reported below was to investigate a possible effect of atabrine on the vitamin A storage capacity of the liver. Two groups of rats were pair-fed the experimental diet with and without atabrine. A third small group of rats was fed the atabrine-free diet ad libitum. Each rat received 100, 200, or 500 I.U. of vitamin A dissolved in 0.1 ml olive oil 6 times weekly. After two, 4, or 6 weeks the animals of each group were killed and their livers analyzed for vitamin A. The results are given in table 1.

This table shows that in most of the experiments the livers of rats on the atabrine-containing diets stored significantly less vitamin A than those of animals kept on the atabrine-free diet. This effect of atabrine was found with all three levels of vitamin A supplementation. No significant difference in the liver storage of vitamin A was noted in experiments 5 and 6. The time of experiment 5, two weeks only, was perhaps too short for the accumulation of toxic amounts of atabrine in the rats. The difference observed in experiment 6, though rather great, was also found to be

statistically not significant owing to the great variations of the vitamin A values of the livers in group B. As was to be expected, no marked differences in liver vitamin A were found between the two groups fed the atabrine-free diet, the one in restricted amounts and the other ad libitum.

For assessment of the vitamin A storage in the liver the liver storage test of Guggenheim and Koch ('44) was em-

TABLE 1

The effect of atabrine on the storage of vitamin A in the liver[1]

NO. OF EXP.	DAILY DOSE OF VITAMIN A	DURATION OF ATABRINE TREATMENT	VITAMIN A PER LIVER, I.U.			P[2]
			A	B	C	A vs. B
	I.U.	*weeks*				
1	100	4	31 ± 9.7 (6)	67 ± 12.1 (6)	69 ± 13.0 (3)	0.01
2	100	6	54 ± 9.1 (3)	97 ± 9.5 (3)		0.01
3	200	4	70 ± 6.1 (6)	131 ± 14.3 (6)	120 ± 11.5 (3)	0.01
4	200	6	86 ± 12.2(3)	127 ± 13.3 (3)		0.01
5	500	2	635 ± 493 (6)	660 ± 145 (6)		0.9
6	500	4	1257 ± 272 (6)	2140 ± 1249 (6)		0.3
7	500	6	1840 ± 442 (12)	2880 ± 289 (12)		0.01

[1] Vitamin A given in daily doses during atabrine treatment. The figures represent means and standard deviations. The values in parentheses indicate the number of animals.

A: Experimental diet with atabrine, given ad libitum.

B: Experimental diet without atabrine, pair-fed with A.

C: The same diet as B, given ad libitum.

[2] Probability that the mean difference observed between A and B was due to chance.

ployed. The vitamin A content of the liver after dosage has been compared to that produced by standard doses of vitamin A. Four groups of rats containing 9 to 13 animals were maintained on experimental diets. Two groups received atabrine, the two remaining groups serving as controls. After 4 weeks the test was performed with carotene as well as with vitamin A and similar results were obtained.

Using as 100% the value for vitamin A in the control animals, a biological value of 58% for vitamin A was found in

the atabrine-treated rats. Carotene gave a value of 44% in normal rats, compared to 32% in animals given atabrine. The value of 44% for carotene in normal animals compares well with that obtained in a previous investigation (Guggenheim, '44).

In a further set of experiments 24 young rats received on 4 consecutive days 25,000 I.U. or 50,000 I.U. of vitamin A per day. After the last dose the rats were placed on the experimental diets with and without atabrine. After two and 4 weeks three rats of each group were killed and their liver vitamin A determined. The results are given in table 2.

TABLE 2

The effect of atabrine on the storage of vitamin A in the liver[1]

DURATION OF EXPERIMENT	AMOUNT OF VITAMIN A GIVEN PREVIOUS TO ATABRINE TREATMENT	VITAMIN A FOUND IN LIVER		
		A	B	A as % of B
weeks	*I.U.*	*I.U.*	*I.U.*	
2	4 × 25,000	5,170	5,780	89
4	4 × 25,000	1,030	2,500	41
2	4 × 50,000	17,600	18,600	95
4	4 × 50,000	10,700	13,400	72

[1] Vitamin A given in 4 daily doses previous to atabrine treatment. The figures represent the averages of three pooled livers.

A: Experimental diet with atabrine, given ad libitum.

B: Experimental diet without atabrine, pair-fed with A.

It can be seen in table 2 that atabrine depletes the stores of vitamin A which have been accumulated in the liver before the treatment. It can also be seen that a two-week treatment was not sufficient to affect the vitamin A content of the liver significantly. After 4 weeks a marked difference was noted.

Vitamin A depletion

The decreased accumulation and rapid disappearance of vitamin A in the liver during atabrine treatment may be due to increased endogenous destruction or to a diminished storage capacity of the liver or to both of these effects. If vitamin

A is metabolized more rapidly in the body, the requirement for the vitamin should be increased. In order to test this hypothesis, three groups of young rats, each comprising 10 animals, were placed on the experimental diets. Group A was atabrine-treated, whereas groups B and C were fed the atabrine-free diet. Group C received twice weekly 100 I.U. vitamin A, whereas the other groups did not receive any vitamin A supplement. The rats of groups B and C were offered the same quantity of food as was eaten by group A, which was, on the average, 4.9 gm per day. After 7 weeks on these diets the following mean weight increases were noted: group A, 49 gm; group B, 48 gm; group C, 59 gm. The small amounts of vitamin A present in the rats at the beginning of the depletion period had already been used by the rats in the first weeks of depletion, before the atabrine in the body reached a toxic and vitamin A-destroying level. It appears, therefore, that atabrine administration does not increase the body's requirement for vitamin A.

Absorption of vitamin A and of carotene

It seems, however, that the toxic influence of atabrine on vitamin A metabolism is not limited to diminishing the storage capacity of the liver for vitamin A. A distinct effect on the absorption of vitamin A and of carotene could also be demonstrated. In these experiments rats which had been maintained for 5 to 6 weeks on the atabrine-containing experimental diet were employed. Each rat was fed 3,300 I.U. of vitamin A or 2.2 mg of carotene, dissolved in 1.0 ml of olive oil. The animals were killed after 9, 12, or 15 hours, and their intestinal contents, intestinal walls and livers analyzed for vitamin A and carotene. Control rats fed the atabrine-free diet were similarly treated. The results are shown in table 3.

As can be seen from table 3, the amount of carotene and of vitamin A found in the intestinal contents of the rats previously fed the atabrine-containing diet was considerably higher than that in the controls; accordingly the quantities

of vitamin A recovered from the intestinal walls were markedly lower. A very great difference could be detected in the vitamin A content of the livers, those of the atabrine-fed animals containing only 20 to 40% of the amount of vitamin A found in the controls.

TABLE 3

The effect of atabrine on the absorption of vitamin A and of carotene

CAROTENOID GIVEN	TIME AFTER ADMINISTRATION OF THE CAROTENOID	ATABRINE	NO. OF RATS	INTESTINAL CONTENT		INTESTINAL WALL, VITAMIN A	LIVER, VITAMIN A
				Carotene	Vit. A		
	hours			*mg*	*I.U.*	*I.U.*	*I.U.*
Vit. A, 3,300 I.U.	9	+	3	...	1,125	19	44
Vit. A, 3,300 I.U.	9	—	3	...	430	25	157
Vit. A, 3,300 I.U.	12	+	2	...	680	36	170
Vit. A, 3,300 I.U.	12	—	2	...	245	51	610
Vit. A, 3,300 I.U.	15	+	3	...	63	39	570
Vit. A, 3,300 I.U.	15	—	3	...	30	46	1,330
Carotene, 2.2 mg	15	+	2	0.47	..	81	20
Carotene, 2.2 mg	15	—	3	0.37	..	93	113

DISCUSSION

Our experiments show that prolonged administration of atabrine exerts a profound effect on the metabolism of vitamin A. Atabrine damages the liver, impairing its ability to store vitamin A and thus depriving the animal of its relative independence of variations in the supply of the vitamin obtained from its food. Furthermore, it seems to depress the absorption rate of vitamin A and of carotene. After administration of the provitamin, vitamin A could be detected in the intestinal wall, thus confirming the work of earlier investigators (Sexton et al., '46; Glover et al., '48; Kon and Thompson, '51), who reported that the conversion of carotene into vitamin A takes place in the intestinal wall. The vitamin A requirement does not appear to be affected by atabrine. A similar conclusion was arrived at by Hegsted, McKibbin

and Stare ('44a), who fed rats suboptimum levels of carotene and did not observe a marked growth inhibition due to atabrine. The level of atabrine fed by these authors, 400 mg per kilogram of ration was, however, considerably lower than that used in the present experiments.

In spite of its toxic effect on vitamin A metabolism, the atabrine level employed by us did not impair the utilization of food. The reduced growth of the atabrine-fed rats was due only to the diminished food intake, probably resulting from the bitter taste of the drug. With a synthetic diet composed of casein, starch, olive oil, minerals and vitamins, a considerable impairment of food utilization could be observed if the diet contained 800 mg atabrine per kilogram of ration (unpublished results). Ershoff ('50) has shown that the addition of desiccated liver or yeast to a synthetic diet counteracts the toxic effects of prolonged atabrine administration. It is possible that milk powder contains also the protective factor or factors which are required in increased amounts by the atabrine-fed rat.

SUMMARY

1. Atabrine added to a diet containing 50% skim milk powder at a level of 800 mg per kilogram of ration does not impair the utilization of food by the rat, if adequate supplements of vitamin A are also given.

2. Prolonged treatment with atabrine has an adverse effect on the capacity of the liver to store vitamin A.

3. Even toxic levels of atabrine which decrease the ability of the liver to store vitamin A do not affect the requirement for the vitamin.

4. The absorption rate of carotene and of vitamin A is decreased by prolonged administration of atabrine.

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RANCID LARD EFFECT ON RATS FED COMPLETE AND RIBOFLAVIN-DEFICIENT DIETS [1]

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ONE FIGURE

(Received for publication September 10, 1951)

In earlier studies (Kaunitz and Slanetz, '50a, b), it was demonstrated that, contrary to previous claims, highly rancid lard was well tolerated by rats when liberal amounts of the known essential food factors were administered separately. When, however, vitamin A was omitted from the diet, the deficiency state developed very rapidly (Stoerk, Kaunitz and Slanetz, '52).

In the present examinations, the influence of rancid lard on the development of riboflavin deficiency in rats was studied.

MATERIALS

The basic diet used in these experiments consisted of 54% cerelose, 30% alcohol-extracted casein, 2% celluration, 4% salt mixture (U.S.P. 2), and 10% rancid lard, and was supplemented, per kilogram, by 1,000 mg of inositol, 1,000 mg of choline, 300 mg of *p*-aminobenzoic acid, and 100 mg of nicotinic acid. Every other day the animals received two drops of a watery suspension containing, per milliliter, 4 mg of thiamine chloride, 8 mg of riboflavin, 8 mg pyridoxine, 20 mg calcium pantothenate, 5 mg of folic acid, 0.05 mg of

[1] Aided by a grant from the Williams-Waterman Fund of the Research Corporation.

biotin, and 10 μg of vitamin B_{12}.[2, 3] Twice weekly they were fed three drops of a linoleic acid suspension containing, per milliliter, 5 mg of crystalline beta carotene,[4] 50 mg of alpha-tocopherol acetate, 10 mg of free alpha-tocopherol, and 0.5 mg of calciferol.[5] This was done in view of the destructive effect of rancid lard on vitamins (Burr and Barnes, '43; Quackenbush, '45).

Albino rats of the Sherman strain, inbred for 15 generations, were used. Mothers were placed, within two days of the birth of their litters, on a diet similar to that given above, which contained lactalbumin and cottonseed oil[6] instead of casein and lard. When the young were 21 to 24 days old, they were placed on the rancid lard diet. At 28 to 30 days, the feeding experiments were started with groups of paired litter mates of the same sex having average weights identical at 21 days and again at 28 days.

In the paired feeding experiments the groups were made up of individually matched animals, and the unrestricted food intake of each animal on the rancid lard diet was determined daily. The corresponding animals in the other groups received the same amount of diet one day later.

The fat used in the diets was either rancid or non-rancid lard. Lard was made rancid by aeration at 90°C. Two samples were prepared, one of which had been treated for 300 hours and the second for 30 to 50 hours. Peroxide number determinations gave values of 200 to 300 in the earlier stages of the aeration, but the values decreased as the aeration was continued — probably because the peroxides polymerized or decomposed.

[2] Doctor Leo A. Pirk of Hoffmann-La Roche, Inc., generously supplied us with most of the vitamins used.

[3] Vitamin B_{12} was supplied through the courtesy of Dr. Augustus Gibson of Merck and Company.

[4] Courtesy of the Barnett Laboratories.

[5] Courtesy of Dr. M. L. Tainter of the Sterling-Winthrop Research Institute.

[6] Wesson.

In the early part of the work a molecular distillate [7] of freshly rendered lard was used as the non-rancid fat (Kaunitz and Slanetz, '51). Later, the lard left after the distillation proved to be equally suitable. These experiments could therefore be combined.

EXPERIMENTAL

In table 1 (a) are given data concerning the growth and food consumption of 7 groups of rats receiving the purified, complete diet with either rancid or non-rancid lard. The food consumption was determined for several weeks and the average was used for the table. The first three groups received strongly rancid lard and the remaining ones mildly rancid lard. It is evident that, regardless of the degree of rancidity, the food consumptions of the groups on rancid and non-rancid lard showed only negligible differences.

The weights of the groups were, however, somewhat different. One group of the rats receiving strongly rancid lard grew better than those on the non-rancid lard diet. Twice, however, the opposite was the case. In the mildly rancid lard experiments the differences were small, ranging from 2 to 6% of the body weight. Nevertheless, in view of the uniformity of the difference, one might assume that the intake of rancid lard led to slightly reduced growth even if all known factors were supplied. This slight difference in growth did not seem to bring on any functional or histological changes in the rats on rancid lard diets (Stoerk, Kaunitz and Slanetz, '52). We succeeded in breeding 5 generations of rats which had received rancid lard as the only fat.

In figure 1 are shown weight and food intake measurements on two groups of riboflavin-deficient rats on either rancid or non-rancid lard. Among the animals on rancid lard, severe growth retardation was soon evident and their food intake gradually declined. When riboflavin was again administered, their weight and food intake increased rapidly,

[7] Distillation Products Industries.

demonstrating that both the reduced food intake and the weight deficit were true consequences of the riboflavin deficiency state.

Nine similar experiments with riboflavin-deficient animals are summarized in table 1 (b). When strongly rancid lard

TABLE 1

Growth and food consumption of rats receiving 10% rancid or non-rancid lard

NUMBER IN EACH GROUP	TIME ON DIET	AGE AT TERMINATION	RANCID LARD		NON-RANCID LARD		
			Average body weight	Average daily food consumption	Average body weight	Average daily food consumption	Weight difference
	days	*days*	*gm*	*gm*	*gm*	*gm*	*%*
			a. Complete diet				
3[1]	46	79	277	13.1	248	13.2	— 12
4[1]	49	82	174	11.4	194	10.7	10
4[1]	56	89	238	12.5	287	12.8	17
4[2]	56	89	271	13.2	287	12.8	6
4[2]	43	76	216	11.3	221	10.8	2
4[2]	26	55	191	11.0	202	11.1	5
4[2]	49	82	186	11.8	194	10.7	4
			b. Riboflavin-deficient diet				
6[1]	72	100	80	...	138	...	42
4[1]	49	77	75	...	146	...	49
4[1]	66	96	154	8.9	209	11.7	26
4[1]	66	96	106	6.4	172	10.3	38
4[1]	52	82	120	7.0	180	9.8	33
4[1]	49	82	137	8.5	199	10.8	31
4[2]	49	82	150	9.6	188	9.9	20
4[2]	43	76	111	8.9	132	7.2	16
4[2]	26	55	133	7.6	171	9.8	22
			c. Complete diet, paired feeding				
6[1]	26	54	128	8.8	129	8.8	1
5[2]	56	85	167	9.2	177	9.2	6
3[2]	23	71	159	7.6	166	7.6	4
			d. Riboflavin-deficient diet, paired feeding				
6[1]	26	55	61	4.1	73	4.1	16
6[2]	56	85	94	5.3	101	5.3	7
6[2]	23	71	141	6.8	156	6.8	10

[1] Lard: 300 hours' aeration.

[2] Lard: 30 to 50 hours' aeration.

[3] With 25% mildly rancid lard and 2% linoleic acid.

was used (first 6 groups), the weights and food intakes were always greatly reduced in comparison to those of rats receiving non-rancid lard. On mildly rancid lard less pronounced weight differences persisted, although no differences in food intake were observed. Inasmuch as the food intakes

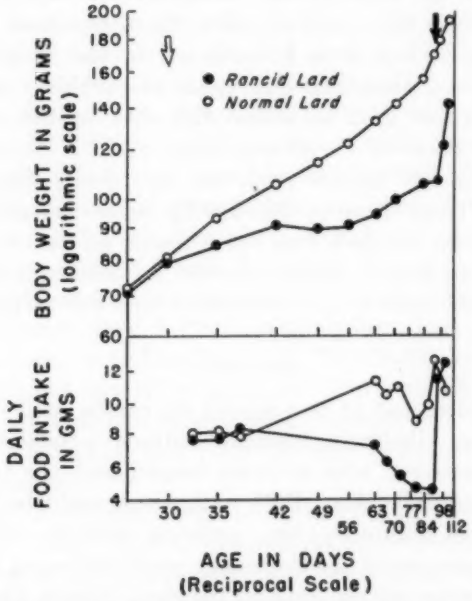

Fig. 1 Average growth and food intake of two groups of 4 riboflavin-deficient, paired litter-mate rats, one fed rancid, the other non-rancid lard. The open arrow indicates when riboflavin was withdrawn; the closed arrow, when it was again supplied.

of the animals on mildly rancid lard and those of their controls on non-rancid lard were identical, but the body weights of the former were lower, it can be concluded that the utilization of the rancid lard diet was less complete than that of the ration containing non-rancid lard. One may infer from this that the deficiency state of the animals on rancid lard was more pronounced, because poor food utilization is one

characteristic of riboflavin deficiency (Sure and Dichek, '41; Mannering and Elvehjem, '44a).

In order to rule out completely the remote possibility that the above results may, in some way, have been influenced by the aversion of the deficient animals to the rancid lard diet, paired feeding experiments were carried out (table 1, c and d). With the complete diet, the differences in weight were slight. They were pronounced in the animals on the deficient diets, demonstrating again the inability of the animals on rancid lard to utilize this diet in the absence of riboflavin. Some of the groups were given a diet containing 25% fat and 2% linoleic acid, but the results did not differ essentially from those obtained with the diet containing 10% fat. This and the fact that the animals on rancid lard supplied with all known factors showed no deficiency signs demonstrate that linoleic acid deficiency was not responsible for the results.

DISCUSSION

The replacement of non-rancid by rancid lard in the diet of rats on riboflavin-deficient rations produced stunted growth; moreover, with strongly rancid lard, the food intake decreased considerably. Both symptoms could be prevented by riboflavin administration, proving that the effects were true consequences of the deficiency state and were not caused by the aversion of the rats to the diet. It can therefore be concluded that the strongly rancid lard accentuated the deficiency state. This conclusion is supported by studies of the food intake of riboflavin-deficient rats (Sure, '41; Voris et al., '42), in which it was found that riboflavin deficiency hardly reduced the appetite of rats unless they were in an advanced stage of the disease. The animals on midly rancid lard were in an intermediate stage of the deficiency, because their weight was higher than that of the group on strongly rancid lard but lower than that of the group on non-rancid lard, although the food intakes of the rats on mildly rancid and on non-rancid lard were practically the same.

In an attempt to analyze the mechanism of this effect, the riboflavin content of the non-rancid and rancid fats was examined. No difference of any significance was found, the analyses yielding values of 0.10 to 0.17 μg per gram. All diets were, therefore, equally riboflavin-deficient.

A clue for further studies was furnished by the observation that riboflavin is produced by the rat's intestinal flora, particularly in the cecum (Mannering et al., '44b). Two pair-fed groups of 6 rats each received either 25% of the mildly rancid or 25% of the non-rancid lard in their diet. After 23 days on the diet the animals were sacrificed and the riboflavin content of the cecal feces was determined. It was found to be, in the non-rancid group, only once below 4 μg per gram of wet feces, but only once above 4 μg in the rancid group, the averages being 4.2 and 3.6, respectively — just on the borderline of statistical significance.[8] Although these preliminary studies need further confirmation, they do suggest that one of the possible causes of the rancid lard effect is the reduction in available intestinal riboflavin. Whether this is due to destruction or depressed production, or whether rancid lard is lacking in a principle stimulating the intestinal flora, is uncertain.

Riboflavin deficiency is probably not the only condition intensified by rancid lard. When fed to rats on vitamin A-low diets, it causes rapid development of the associated syndrome (Kaunitz and Slanetz, '50a; Stoerk, Kaunitz and Slanetz, '52). Similarly, preliminary experiments show that the same may be true for thiamine deficiency. Rancid lard, therefore, while adequate as a fat when supplemented by liberal amounts of the essential factors, leads to the rapid development of deficiency symptoms when the factors are supplied at low levels. This effect probably explains the many observations of the occurrence of a fatal disease in rats on rancid lard diets. The inclusion of the vitamins in a rancid lard diet

[8] The riboflavin analyses were carried out by the Laboratory of Industrial Hygiene, New York City, according to the microbiological method, U. S. P. XIV.

leads to the damage of some of the factors while, at the same time, the requirements for these factors are increased. It is not impossible that this could be of advantage for experimental work, in that the inclusion of rancid lard in experimental diets may speed up the development of a deficiency state.

The effect of rancid lard may offer a clue as to some of the non-caloric properties of normal fats, especially as to why the inclusion of a good fat in diets is necessary to ensure optimum results. The hypothesis seems plausible that normal fats (but only at proper levels) minimize the occurrence of known and still unknown deficiency diseases, along with all of the consequences of the latter, such as weight deficits, failure of fertility, infections, and so forth.

This action of good fats may, in some instances, be due to the presence of factors capable of replacing a known vitamin, such as the "lard factor" which can take the place of vitamin A (Kaunitz and Slanetz, '50a, b). In other instances, it may be due to the capacity of the normal fat to stimulate the production of essential factors by the intestinal flora.

SUMMARY

The inclusion of 10% of rancid lard in a purified rat diet led to no toxic symptoms when all known essential factors were fed separately. Five generations of rats were bred with rancid lard as the only fat source; animals receiving rancid lard were, however, roughly 5% lighter than their controls.

When riboflavin was withdrawn from rats on strongly rancid lard, severe reduction of growth and food intake occurred, both symptoms disappearing when riboflavin was again supplied. A less pronounced effect as to growth retardation was observed in rats on mildly rancid lard. The differences in growth persisted in paired feeding experiments. Rancid lard, therefore, accentuated the signs of riboflavin deficiency. Preliminary experiments indicated that this effect might be due to a reduction in riboflavin available from the intestinal flora.

Rancid lard seems to increase the requirements for various essential factors; this, in addition to its destructive effect on vitamins in the diet, may explain the observations that rancid lard diets lead to a fatal disease.

The hypothesis is put forward that some of the non-caloric properties of normal fats are due to their ability to reduce the requirements for known and still unknown factors, in that they either supply substitutes for these factors or stimulate the production of these factors by the intestinal flora.

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EFFECT OF SINGLE AMINO ACID EXCESSES ON GLUCOSE METABOLISM AND CHICK GROWTH, AS INFLUENCED BY THE DIETARY AMINO ACID BALANCE [1]

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(Received for publication September 24, 1951)

The growth rate of chicks receiving adequate amounts of all known vitamins is decreased by the addition of an excess of certain amino acids to their diet. Anderson et al. ('51) found that DL-methionine depressed the growth rate to a greater extent than any other amino acid. Growth depressions were noted also when high levels of DL-lysine HCl, L-tyrosine, or certain other amino acids were fed. Growth depression in chicks as a result of feeding diets containing an excess of methionine was noted previously by McKittrick ('47), who reported that the depression was overcome by glycocyamine or serine. Wretlind ('49) also found that DL-methionine was the most toxic of all amino acids when fed at high levels to rats. The work of Van Pilsum and Berg ('50) and Wretlind and Rose ('50) indicates that excesses of the L-isomer depress growth more than those of the D-isomer. Brown and Allison ('48) fed rats a diet containing 4.8% DL-methionine and found that they lost weight faster than rats pair-fed the same diet without methionine. The addition of arginine to the diet containing methionine resulted in less weight loss. Roth and Allison ('49) found that the addition of glycine or

[1] Scientific paper A338. Contribution 2308 of the Maryland Agricultural Experiment Station (Department of Poultry Husbandry).

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glycine and arginine also counteracted the weight loss resulting from feeding excess methionine.

Grau ('48), Almquist ('49), Almquist and Merritt ('50), and Grau and Kamei ('50) have reported that the dietary level of certain essential amino acids required for rapid growth in the chick is increased as the level of protein in the diet is raised. This suggested that the growth depression produced by feeding high levels of single amino acids might be overcome by providing a more favorable amino acid balance.

The work presented in this paper was done to determine if the growth depression caused by feeding excesses of tyrosine, lysine, or methionine could be overcome by the addition of other amino acids or proteins which would improve the amino acid balance. The results of glucose tolerance tests on chicks fed excess amino acids are also presented.

EXPERIMENTAL

Except as indicated, day-old New Hampshire chicks were distributed into uniform groups of 10 or 20 on the basis of body weights at the beginning of each experiment, and maintained in electrically heated batteries with raised wire floors. Feed and water were supplied ad libitum. Weighings and other observations were made at weekly intervals.

Basal diet 112, described by Anderson et al. ('51), was used in most of the experiments. It contains 66.4% glucose (cerelose), 18% crude casein, 5% gelatin, 4% soybean oil, 0.3% DL-methionine, minerals, and vitamins. In experiment 4 this diet was modified to contain 24% crude casein at the expense of glucose. Amino acids replaced glucose when added to these diets. A complete practical-type, all-mash ration was employed as the basal ration in other experiments. When amino acids were added to this ration, they replaced an equal amount of mash.

Where glucose tolerance tests were done, 3 gm of glucose per kilogram of body weight were administered orally in a 30% water solution. A blood sample was taken just before giving glucose; another, one hour later; and a third, two and

one-half or three hours later, as indicated. Six representative birds of each group were used for each test. The first blood sample was taken from 4 birds. Two of these birds were used to obtain the second sample and two for the third. Blood was drawn from the two remaining birds for the second and third samples. Only two 2-ml samples were removed from each chicken, since preliminary studies revealed lower blood glucose levels in the last sample when three samples were removed from the same bird.

RESULTS AND DISCUSSION

Amino acid balance and growth rate

The first three experiments were conducted to determine the effect of amino acid balance on the growth-depressing effect of high levels of DL-lysine HCl and L-tyrosine. Liver meal, gelatin, zein, and ground corn were added alone and in combination to diets containing normal or high levels of lysine or tyrosine. The results are given in table 1. In experiment 1, the growth rate of chicks which received diet 112 plus 4% DL-lysine HCl was only 36% of that of chicks fed the unsupplemented diet (112). This level of lysine caused such a marked depression in growth that the level was reduced to 3% in the second experiment.

When 3% liver meal or 5% gelatin was added to diet 112, the average gain of chicks receiving the high level of DL-lysine HCl was 64 and 72% as great, respectively, as that of chicks fed the same diets without the added lysine. The addition of 3% liver meal to diet 112 plus 5% gelatin was of no further value in overcoming the growth-depressing action of lysine. However, the addition of either 5% zein or 25% ground corn resulted in less depression. In experiment 3, the addition of 4% L-tyrosine to diet 112 also resulted in a marked decrease in growth. This growth depression was almost entirely overcome by the inclusion of 5% gelatin in the diet.

Gelatin contains little or no tyrosine but does contain lysine and a comparatively high level of arginine. Other experi-

ments indicate that the limiting amino acid in diet 112 is arginine. Zein contains little or no lysine, and corn is also low in lysine. The addition of gelatin, corn meal, and zein to the diet containing 3% lysine and the addition of gelatin to the diet containing 4% tyrosine increases the levels of

TABLE 1

Effect of various protein supplements on the growth-depressing action of DL-*lysine HCl and* L-*tyrosine*

EXPERIMENT NO.	SUPPLEMENT TO DIET 112	AVE. WEIGHT (4 WK.)		INCREASE IN GAIN [2]
		Without added lysine	With added lysine [1]	
		gm	*gm*	%
1	None	260(20)[3]	120(18)	36
2	3% liver meal	272(10)	189(8)	64
	5% gelatin	332(9)	248(9)	72
	5% gelatin + 3% liver meal	350(9)	251(10)	68
	5% gelatin + 5% zein	320(10)	272(8)	83
	5% gelatin + 3% liver meal + 25% corn	404(10)	327(10)	79
		Without added tyrosine	With added tyrosine [1]	
3	None	183(8)	115(10)	53
	5% gelatin	287(10)	270(9)	93

[1] In experiment 1, 4% DL-lysine HCl was added, while 3% was added in experiment 2. L-tyrosine was added at a level of 4% in experiment 3.

[2] Weight gain of chicks fed excess lysine or tyrosine divided by weight gain of chicks fed the same diet without the added amino acids. The average initial weight of chicks was approximately 40 gm.

[3] Figures in parentheses refer to number of surviving chicks. In experiment 1, 20 chicks were used per group. Ten chicks were used per group in the other two trials.

other amino acids. Thus, it appears that the growth depression resulting from the addition of excess lysine or tyrosine is counteracted by providing a more favorable amino acid balance.

Reports cited above suggested that the growth depression caused by feeding high levels of methionine might be over-

come by increasing the level of arginine or arginine and glycine in the diet. Consequently, experiment 4 was conducted to study this relationship. The results are given in table 2. The basal diet contained 24% crude casein and 60.4% cerelose but, otherwise, was similar to diet 112. If the arginine supplied by casein is available to the chick, this diet furnished 1.28% arginine, which is adequate for the rapid growth of chicks fed a 20% protein diet. The growth was very poor unless additional arginine was supplied. The addition of 0.5% arginine HCl was adequate in this trial. This diet contained 26.5% protein, which would increase somewhat the level of arginine required for rapid growth. Nevertheless, this observation is similar to those made by Arnold et al. ('36) and Klose et al. ('38), which suggested that the arginine present in casein is not completely available to the chick. The growth of chicks fed the diet containing 3% additional DL-methionine was less than half that of chicks fed the basal diet. This growth depression was only slightly influenced by the addition of arginine or gelatin to the diet, even though a more desirable amino acid balance was provided.

TABLE 2

Effect of arginine and gelatin on the growth depression of chicks resulting from high levels of dietary DL-*methionine*

ADDITION TO DIET (112 + 6% CASEIN)	AVE. WT. AT 4 WK.		INCREASE IN GAIN [1]
	Without added methionine	With 3% added DL-methionine	
	gm	*gm*	%
None	160(10)[2]	89(6)	41
0.5% L-arginine HCl	319(10)	92(5)	19
1.0% L-arginine HCl	280(9)	133(9)	39
2% L-arginine HCl	330(10)	111(8)	24
5% gelatin	309(10)	123(10)	31

[1] Weight gain of chicks fed excess methionine divided by weight gain of chicks fed the same diet without added methionine. The average initial weight of chicks was approximately 40 gm.

[2] Figures in parentheses refer to number of surviving chicks. Ten chicks were used per group at the start.

This may be explained on the basis of poor feed consumption. Chicks fed diets containing added methionine consumed very small amounts of feed. In other experiments, chicks fed diets containing more than 3% methionine consumed even less feed (force-feeding of a greater amount of a ration with excess methionine was not done). Experiments were conducted in which chickens were force-fed a glucose-methionine mixture. These chickens lost significantly less weight than chickens force-fed an equal amount of glucose. When a mixture of 95% glucose and 5% DL-methionine was offered ad libitum, the amount of feed consumed was less than one-fourth that consumed by chicks offered glucose only, and the body weight loss was significantly greater. This suggests that the slow rate of growth noted with chicks fed an excess of DL-methionine is due in part to the self-restricted feed intake.

Influence of excesses of amino acids on glucose metabolism

Preliminary experiments revealed that chicks previously fed high levels of methionine had higher blood glucose levels during glucose tolerance tests than chicks fed ordinary rations. This was true even if the excess methionine were withdrawn for as long as 11 days before the glucose tolerance test was conducted. Subsequent to this observation, a number of tests were performed with chicks which previously had been fed a high level of different amino acids. Similar tests were also done with chicks fed diets without added amino acids. Addition of high levels of these amino acids to both practical and purified-type diets produced similar changes in the blood glucose levels. The results of three typical experiments are given in table 3. In these tests, chickens fed amino acid excesses had higher blood glucose levels 60 minutes after being given glucose than chickens fed the same diet without the added amino acid. Only two exceptions were

noted; both of these were with chickens previously fed DL-lysine HCl.

Five glucose tolerance tests were done with chickens previously fed a high level of DL-methionine. In each instance,

TABLE 3

Effect of high dietary levels of single amino acids on blood glucose level found in glucose tolerance test

EXPERIMENT NO.	ADDITION TO COMPLETE PRACTICAL RATION	SIX-DAY GAIN	BLOOD GLUCOSE LEVEL AT			
			0 min.	60 min.	150 min.	180 min.
		gm		*mg %*		
5	None	86(6)[2]	172	228	192	
	5% glycine	36(6)	177	264	215	
	5% L-leucine	50(6)	190	246	207	
	5% DL-lysine HCl	28(6)	174	214	215	
	5% DL-methionine	—42(6)	186	226	242	
6	None	160(6)	171	199		171
	None (methionine in test)[1]	144(6)	168	187		177
	5% DL-methionine	—38(6)	171	216		202
	5% DL-methionine (methionine in test)[1]	—84(6)	167	254		202
	ADDITION TO DIET 112-NIACIN:	FOUR-WK. WT.				
7	None	191(12)	165	191		190
	10 mg % niacin	279(12)	168	189		186
	4% DL-phenylalanine	126(10)	..[3]	214		229
	4% DL-phenylalanine and 10 mg % niacin	153(6)	196	..[3]		296
	4% L-arginine HCl	119(6)	146	249		208
	4% glycine	166(9)	163	232		172
	4% glycine and 10 mg % niacin	302(11)	..[3]	234		208
	4% DL-lysine HCl	148(11)	176	231		304

[1] One hundred and fifty milligrams per kilogram of body weight.
[2] Figures in parentheses refer to the number of chicks.
[3] Blood sample lost.

the blood glucose levels did not return to the fasting level as rapidly as they did when the chickens were fed the same diet without added methionine. This was also true of chickens which had previously been fed diets containing either

4 or 5% DL-lysine HCl or 4% DL-phenylalanine. The blood glucose levels of chickens fed diets containing an excess of glycine, arginine, or leucine returned toward the fasting level more rapidly than those of chickens fed ordinary diets or diets containing an excess of lysine or methionine. It is apparent that the feeding of excess lysine, methionine, or phenylalanine influences the glucose metabolism in the chicken.

In experiment 6, given in table 3, the chickens of two groups were given 150 mg of DL-methionine per kilogram of body weight with the glucose solution, but this did not significantly alter the blood glucose levels found later. Apparently this change in glucose metabolism does not develop immediately after the administration of excess methionine and, as indicated previously, it lasts for some time after the excess methionine is withdrawn from the diet.

The manner in which the amino acids influence the metabolism of glucose in the chick is not known. The change in glucose metabolism noted with the chickens fed excesses of methionine, lysine, or phenylalanine may be related in part to the growth depression which resulted.

SUMMARY

Experiments were conducted to determine if the growth depression in chicks caused by feeding a high level of DL-lysine HCl, L-tyrosine, or DL-methionine could be overcome. The growth depression caused by the addition of lysine was proportionally less when zein, corn, or additional gelatin was included in the diet. The growth depression caused by excess tyrosine was also less when additional gelatin was added. These proteins increase the level of other amino acids in the diet and thus appear to provide a more favorable amino acid balance.

The growth depression caused by feeding excess methionine was not influenced by adding arginine or gelatin, even though these make a better dietary amino acid balance. The restricted feed intake of these chicks is believed to be largely

responsible for the growth depression caused by excess methionine.

Chicks fed high and normal levels of various amino acids were subjected to glucose tolerance tests. When the chicks were fed diets high in DL-methionine, DL-lysine HCl, or DL-phenylalanine, the blood glucose level following sugar ingestion did not return to normal as rapidly as occurred when chicks received normal diets or diets high in L-arginine HCl, L-leucine, or glycine.

ACKNOWLEDGMENTS

This work has been supported in part by a grant from E. I. du Pont de Nemours and Company, New Brunswick, New Jersey.

The authors are indebted to Merck and Company, Inc., Rahway, New Jersey, for crystalline vitamins; Wilson and Company, Inc., Chicago, Illinois, for gelatin; Allied Mills, Inc., Portsmouth, Virginia, for soybean oil; Abbott Laboratories, North Chicago, Illinois, for haliver oil; U. S. Industrial Chemicals, Inc., New York, New York, for DL-methionine; Lederle Laboratories, Pearl River, New York, for folic acid; and E. I. du Pont de Nemours and Company, New Brunswick, New Jersey, for irradiated 7-dehydrocholesterol and DL-lysine HCl.

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NUTRITIONAL VALUE OF PLANT MATERIALS

VII. DIETARY FACTORS AFFECTING PRE-WEANING AND POST-WEANING MORTALITY OF RATS[1]

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(Received for publication September 24, 1951)

Previous studies revealed a high incidence of a fatal acute uremia of newborn rats whose mothers were fed rations low in vitamin B_{12} and containing either 24% of protein in the form of a commercial soybean sodium proteinate or mixtures of soybean oil meal, yellow corn, and alfalfa leaf meal containing from 15.6 to 21.8% of protein (Halvorson and Schultze, '50). With the soybean protein diet the external symptoms of acute uremia and the high early mortality of the young could be prevented by such diverse means as: (1) administration of liver extract or fish solubles to the mothers or of vitamin B_{12} to the young (Schultze, '49; Halvorson and Schultze, '50); (2) extensive purification of the soybean sodium proteinate (Schultze, '50a); (3) inclusion of 2% of sulfathalidine in the maternal ration (Schultze, '50b).

Further studies on the effects of maternal nutrition on the incidence of acute uremia and mortality in the young were therefore clearly indicated. A high protein content in the diet increases the vitamin B_{12} requirement of the rat (Hartman et al., '49; Sure, '51). In this study observations were therefore made of the mortality of young rats whose mothers consumed vitamin B_{12}-low diets containing soybean oil meal. These diets were modified by: (1) increase of the protein content; (2) deletion of added methionine; (3) addition of alfalfa

[1] Paper 2697, Scientific Journal Series, Minnesota Agricultural Experiment Station.

leaf meal; (4) addition of alfalfa leaf meal and yellow corn; (5) addition of vitamin B_{12} to diets producing a high incidence of mortality in the young.

EXPERIMENTAL

Animals and rations

The rats were F_3–F_4 generation offspring of animals described in an earlier paper (group 2, Schultze, '50c). The intervening generations had never been fed a known source of vitamin B_{12} activity. Four or 5 rats were housed together in cages provided with raised screened bottoms, except during the last few days of pregnancy and during lactation, when they were kept in individual cages containing clean wood shavings. The males used for breeding were fed the same rations as the females. The rats were bred when they were 10 weeks old or weighed 150 gm, whichever occurred later. The animals of groups 1, 2, 3, 8, and 9 were maintained on the rations indicated from weaning and throughout at least one reproductive cycle. Since previous experience had shown that some changes in the maternal ration are quickly reflected in the survival of the young, the animals of group 1 were transferred in succession to group 5, then 6, then 7, after weaning or loss of a litter. This permitted observations on the progressive effects on the mortality of the young of rations containing soybean oil meal with added methionine, without added methionine, with alfalfa leaf meal, and with alfalfa leaf meal and yellow corn. Similarly, after each of the rats in group 2 had weaned or lost three or 4 litters, their diet (ration S_9, 33% protein as soybean oil meal) was supplemented with 40 μg of vitamin B_{12} per kilogram (ration S_{10}, group 4). The composition of the rations is shown in table 1. Except for rations CS–1 and CS–4, vitamins, including biotin, were supplied as the mixtures previously described (Schultze, '50c), with synthetic vitamin A acetate replacing the concentrate formerly used.

TABLE 1

Composition of rations

CONSTITUENTS	RATION							
	S_7	S_9	S_{10}	S_{11}	S_{12}	CS-3	CS-1	CS-4
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Soybean oil meal, expeller	485.0	728.0	728.0	485.0	427.0	375.0	175.0	175.0
Alfalfa leaf meal	..	..	..	..	150.0	50.0	150.0	150.0
Ground yellow corn	..	..	..	..	..	533.4	753.4	753.4
Salt mixture [1]	40.0	40.0	40.0	40.0	40.0	..	..	..
Salt mixture [2]	..	..	..	..	..	21.6	21.6	21.6
Sucrose	369.4	126.4	126.4	375.0	277.4	..	..	..
Sucrose + B vitamins [1]	20.0	20.0	20.0	20.0	20.0	20.0	..	..
Vegetable fat (Crisco)	70.0	70.0	70.0	70.0	70.0	..	..	..
Corn oil + vit. A, D_3, E [1]	10.0	10.0	10.0	10.0	10.0	..[4]	..[4]	..[4]
DL-methionine	5.6	5.6	5.6	..	5.6	..	..	..
Vitamin B_{12} [3]	..	..	40 μg/kg	..	..	..	..	40 μg/kg

[1] Schultze ('50c).
[2] Spitzer and Phillips ('46).
[3] Merck and Co., 0.1% triturate of vitamin B_{12} in sodium chloride.
[4] Vitamins A and D supplied by two drops of haliver oil (Abbott) per week.

RESULTS AND DISCUSSION

Incidence of symptoms of acute uremia

Table 2 summarizes the results. Since the primary objective was to observe the effects of maternal diet on the survival of the young, none of the young was sacrificed for determination of blood urea. The estimate of the incidence of acute uremia is based, therefore, on observation of the typical symptoms described earlier (Schultze, '49). It is recognized that some individuals may have a relatively high concentration of blood urea while appearing normal (Liener and Schultze, '50). The present data show the following relations between incidence of symptoms of acute uremia of the young and composition of the maternal diet: (1) the incidence was zero when the diet was low in vitamin B_{12} and contained about 22% of protein from soybean oil meal with or without

TABLE 2

Effect of maternal diet on mortality of young

GROUP	1	2	3 [4]	4	5	6	7	8	9
Ration: No.	S_7	S_9	S_9	S_{10}	S_{11}	S_{12}	CS-3	CS-1	CS-4
Type [1]	S,M	S,M	S,M	S,M	S	S,A,M	S,C,A	S,C,A	S,C,A
% Protein	22.0	33.0	33.0	22.0	21.4	22.0	21.8	15.6	15.6
Vitamin B_{12}	..	..	..	40 μg/kg	..	..	..	..	40 μg/kg
Sequence of litters	1st	1st–4th	1st–4th	4th–5th	2nd–3rd	3rd–4th	4th–5th	1st–2nd	1st–2nd
Number of mothers	31	32	26	29	31	31	30	20	19
Number of litters born alive	31	102	90	28	33	35	31	37	28
Mean number of young born per litter	9.10	7.48	5.89	7.21	9.46	8.05	7.91	7.00	7.17
Per cent of young born dead	5.3	10.1	11.1	4.0	8.8	4.8	9.5	..	..
Per cent litters with acute uremia	0	4.6	3.8	0	0	2.9	58.0	40.0	0
Mortality of young, 0–4th day, %	14.6	39.2	..	16.0	14.2	13.4	48.9	60.5	6.2
Mortality of young to weaning, % [2]	28.1	59.5	44.7	21.6	35.4	31.2	56.3	77.0	22.1
Mean no. young weaned per litter of survivors	7.38	4.79	4.97	6.61	6.96	6.33	5.88	4.64	5.74
Mean weaning weight per survivor, gm [3]	32.0	27.8	29.5	36.6	26.3	29.3	25.7	42.5 [3]	48.0 [3]
± Standard error of the mean	0.38	0.37	1.1	0.34	0.39	0.34	0.46	0.83	0.58
Number of young observed post-weaning	..	273	293	139	267	176	..	..	..
Mortality 14 days post-weaning, %	..	35.5	44.7	0.72	1.88	4.54	..	..	..
Mean weight gain, 14 days post-weaning, gm [3]	..	31.0	33.4	48.4	31.7	27.1	..	..	..

[1] S = soybean oil meal; M = DL-methionine; A = alfalfa leaf meal; C = yellow corn.

[2] The young of groups 1 to 7 were weaned at 21 days, those of groups 8 and 9 at 30 days of age.

[3] Includes only animals which survived the 14 days' post-weaning.

[4] Offspring of group 2.

added methionine (groups 1 and 5); (2) the incidence was increased to only about 4% by enhancing the vitamin B_{12} requirement of the rats through feeding of a 33% protein diet (groups 2 and 3). This relation was observed through two successive generations. (3) The inclusion of 15% of alfalfa leaf meal into a 22% protein diet increased the incidence of uremia to only 3% (group 6); (4) the feeding of soybean oil meal-alfalfa leaf meal-yellow corn diets of 21.8 or 15.6% protein content induced symptoms of acute uremia in 40 to 60% of the litters (groups 7 and 8). This was the case even with rats whose previous litters had shown a very low incidence (group 7). (5) The feeding of 40 μg of vitamin B_{12} per kilogram maternal diet eliminated the symptoms of acute uremia of the newborn (groups 4 and 9). It is at present not clear why the incidence of fatal acute uremia of the newborn was low on rations containing soybean oil meal or soybean oil meal plus alfalfa leaf meal, and why a high incidence appeared as soon as mothers were transferred to a ration containing soybean oil meal, alfalfa leaf meal and yellow corn. Calculations made from data published in the literature on the amino acid, potassium, sodium, and chloride content of the various rations used in this and a previously reported study (Halvorson and Schultze, '50) failed to reveal one criterion by which maternal rations conducive to a high incidence of acute uremia of the newborn could be differentiated from those on which the incidence was low.

Pre-weaning mortality

The high incidence of early mortality of the young born to mothers fed the vitamin B_{12}-deficient soybean oil meal-alfalfa-corn rations (groups 7 and 8) is mainly accounted for by the acute uremia of the newborn. The cause of death of the young born to mothers on the vitamin B_{12}-deficient, high protein diet (groups 2 and 3) is not certain. In the latter groups the percentage of the young born dead was highest and in the second generation (group 3) the total number of young born per litter was small. Although upon careful visual

inspection the young appeared to be normal and in most cases vigorous at birth, defects established *in utero,* as indicated by Lepkovsky et al. ('51), may have contributed to the high pre-weaning mortality. Insofar as observation of the young permits a conclusion, the quantity of milk secreted by the mothers during the early stages of lactation was normal in all groups of animals. Therefore, a high early mortality in groups 2, 3, 7, and 8 appears to reflect primarily an abnormal composition of the mothers' milk (vitamin B_{12} is no doubt one of the factors involved), superimposed perhaps on defects established in the young during prenatal life. We have never observed evidence of the hydrocephalus and related symptoms which O'Dell et al. ('51) found in many of the young born to rats maintained on a vitamin B_{12}-deficient ration. The addition of vitamin B_{12} to rations responsible for a high early and pre-weaning mortality produced a marked increase in the number and weaning weight of the survivors. Increase of the vitamin B_{12} content of the high protein ration (S_{10}, group 4) and of the low protein soybean oil meal-alfalfa-corn ration (ration CS–4, group 9) greatly reduced the pre-weaning mortality, in spite of the fact that the latter ration is not satisfactory in other respects, as is shown by the low weaning weight of the young at 30 days of age.

Post-weaning mortality

The young born to mothers maintained on the vitamin B_{12}-deficient 33% protein ration incurred a high incidence of mortality during the first two weeks after weaning. Similar observations have been reported by Zucker and Zucker ('48), Borson et al. ('50), and Lepkovsky et al. ('51). Ration S_9, on which this post-weaning mortality was observed, contained added choline and methionine in addition to the quantities of these compounds present in the soybean oil meal. Thus, from a dietary point of view at least, this condition is differentiated from the high incidence of post-weaning mortality described by Griffith ('41) among rats maintained on choline-deficient rations. Vitamin B_{12} was effective in preventing the

post-weaning mortality reported here (group 4, ration S_{10}). In the establishment of this syndrome, the maternal diet again appears to have a decisive effect. Thus, when 48 rats, born and raised to 21 days of age by mothers fed vitamin B_{12}-deficient rations containing either soybean sodium proteinate or a purified soybean protein at a 24% protein level (rations S_2 or S_3, previously described; Schultze, '50c), were then transferred to the 33% protein soybean oil meal ration S_9, there was not a single case of post-weaning mortality. After these rats had been raised to maturity on the same ration, however, 36% of their young died during a 14-day, post-weaning period (group 2). A high protein content in the ration, therefore, enhances the effect of vitamin B_{12} deficiency and this becomes more marked in the second generation. Judged by a different criterion, the same effect can be observed: the 6-week post-weaning weight gains of female rats in groups 1, 2, and 3 were 126.1 ± 2.38 [2] gm (22 rats), 115.9 ± 1.70 gm (35 rats), and 93.8 ± 2.4 gm (42 rats), respectively. Prolonged maintenance of the rats on the vitamin B_{12}-deficient 33% protein diet (ration S_9) did not appear to impair their reproductive capacity permanently. Thus the animals in group 2 — which produced three to 4 litters of young having a high incidence of stillborn and of early post-natal and post-weaning deaths — subsequently, when supplemented with 40 μg of vitamin B_{12} per kilogram of ration, produced young with a greater viability and a much lower death rate (group 4).

Our data also indicate that in vitamin B_{12} deficiency a high protein ration increases the incidence of pre-natal death of the young. Thus, among the animals of group 2, with 107 pregnancies, 4 litters were born dead and 17 litters died shortly after birth. In the second generation of rats maintained on such a ration (group 3), the number of young born per litter was small compared to other groups. In these respects our observations were similar to those of Sure ('51), although the effects of vitamin B_{12} deficiency were not as severe in our case. However, in contrast to the observations

[2] Standard error of the mean.

made by Sure, who used a ration containing 30% protein in the form of a soybean flour and who observed a very high incidence of sterility among female rats, our rats fed the high-protein diet did not fail to conceive. Jaffé ('48–'49) also reported a high incidence of sterility in female rats fed rations which were no doubt deficient in vitamin B_{12}. The addition of methionine to our rations and differences in the composition of the vitamin supplements may account for these discrepancies.

The mature weight and the condition of the mothers during the reproductive period were satisfactory in all groups except group 3, which consisted of second-generation animals maintained on the 33% protein ration. These animals were smaller and did not appear to have the same vitality and muscular tonus as the mothers in the other groups. These studies emphasize the decisive effect which maternal diet exerts on the survival of the young.

SUMMARY

Further observations have been made on factors of maternal nutrition affecting the incidence of deaths associated with symptoms of acute uremia in the newborn rat. This syndrome was observed in 40 to 58% of the litters born to mothers maintained on rations containing soybean oil meal, alfalfa leaf meal, and yellow corn.

The addition of 40 μg of vitamin B_{12} per kilogram to the maternal ration prevented this syndrome.

On rations containing 48 to 72% soybean oil meal or soybean oil meal plus alfalfa leaf meal, the incidence of the acute uremia of the newborn was very low.

Young born to mothers maintained on a 72% soybean oil ration developed a 35 to 45% post-weaning mortality.

The post-weaning mortality of the young could be prevented by the addition of 40 μg of vitamin B_{12} per kilogram of ration.

ACKNOWLEDGMENTS

Financial support for part of this work from the Williams-Waterman Fund and the assistance of Merck and Company, the Abbott Laboratories, the Lederle Laboratories and E. I. Du Pont de Nemours and Company with various supplies are gratefully acknowledged.

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A STUDY OF THE MINIMUM CALCIUM REQUIREMENTS OF ADULT MEN [1, 2]

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THREE FIGURES

(Received for publication October 11, 1951)

Useful dietary standards for any nutrient can be established only after a consideration of two factors: (a) the influence of the level of intake of the nutrient on the health of the individual; and (b) the availability to the individual of the nutrient in question. Failure to consider the second factor may lead to unrealistic recommendations which disillusion those who take them seriously and find them unattainable, and might lead to a distorted agricultural program, waste of resources, and actual losses in food production. Economic status as well as actual food production must be considered important factors in determining the actual food available.

Dietary allowances in the United States can afford to be generous for most nutrients and are generally recognized to be so. When the supplies of certain nutrients are limited, however, more emphasis must be placed upon food economics

[1] Supported in part by a grant-in-aid from the Abbott Laboratories, North Chicago, Illinois, to the Department of Nutrition, Harvard University School of Public Health.

[2] The work in the penitentiary was made possible by the kind permission of the Minister of Justice and by the extremely helpful cooperation of Señor Luis Solari Saco, Director of the Central Penitentiary of Lima, and his associates.

[3] A United States Government Point 4 Agency.

than on "safety allowances." In this case attempts should be directed toward solving the most pressing nutritional problems, since small improvements in intake will yield large dividends in terms of health. This approach requires considerable knowledge of the nutritional state of the people, their food habits, the quantity and quality of food available, the educational system, agriculture, and so forth. Many populations have lived for long periods of time and developed a high degree of culture and ability to do hard physical labor, although their diets appear to be marginal in respect to certain nutrients. More complete studies of such groups should be an important source of basic information in nutritional science and should help in providing solutions to their own problems.

There is considerable concern over the limited milk supplies in most tropical and subtropical countries, where numerous obstacles must be overcome if milk production is to be increased. These programs will be costly, since often the climate, the land, and the degree of technological development are unsuited for the production and distribution of milk. Nutritional benefits must be great to justify the expansion of a dairy industry under such conditions, and careful study by nutritionists as well as agriculturalists is necessary.

In most dietaries the calcium intake is closely related to the milk intake. This paper records a study of the calcium metabolism of a group of adult Peruvian men whose dietary intake of milk had been low over an extended period of time.

EXPERIMENTAL

The 10 volunteers who served as subjects were inmates of the Central Penitentiary of Lima. They ranged from 30 to 56 years of age. A medical history of each revealed no chronic or recent illnesses. No serious abnormalities or overt signs or symptoms of nutritional deficiencies were found on physical examination. A few minor signs were seen which might be attributed to inadequate intake of certain nutrients.

Conjunctival vascularization was found in the majority of the subjects. Five were considered to have greasy skin in the area around the nose and two showed fairly definite hyperkeratosis on the elbows and other parts of the arms. All but three had several carious teeth. The number of missing teeth varied from two to 19; average, 8.5 missing teeth per person. The average weight was 69.9 kg (range, 56 to 89) and the average height was 1.67 m (range, 1.61 to 1.78). None of the men was obese and 6 were rather thin. All were working at their usual jobs in the prison shops.

The subjects had been imprisoned for periods varying from two to 20 years. During this time they had received the prison diet plus occasional food gifts brought in by their families. The usual prison diet was judged to be adequate in calories. It consisted principally of rice, bread, potatoes, spaghetti, and beans. Meat was served once a day in limited amounts. Some vegetables, such as carrots, cabbage, squash, sweet potatoes, and so forth, were served fairly frequently but green leafy vegetables were practically absent from the diet. Fruits other than bananas were also rare. The only milk received was in one serving of chocolate once a week in place of the usual black coffee or tea. While this diet was supplemented with gifts from outside to a variable extent, milk and green vegetables were not common gifts. Fruits, cookies, and so forth, were more usual. This diet was obviously low in several nutrients when compared to the National Research Council's recommended dietary allowances (Food and Nutrition Board, '48). Calcium, riboflavin, animal protein and ascorbic acid were probably the most limiting. Beans and some yellow vegetables would make carotene, thiamine and niacin less of a problem. The prison doctor reported that some symptoms and signs of deficiency diseases were occasionally seen. It is clear, however, that the diet must have been sufficient to maintain the men in reasonable health over long periods of time.

The amount of sunlight irradiation regularly available may have been of some importance in this study. Ordinarily

there is practically no direct sunlight in Lima from May or June to October but full sunlight during the rest of the year. This study was made in February and March and all but one of the subjects, Ro. had daily access to the prison yard for varying periods of time.

A special room in the penitentiary was equipped with a stove, refrigerator, and so forth, and served as a kitchen and dining room. All meals were prepared and consumed here. The diets were measured with standard cups or spoons after preparation or, in some cases, the items were weighed prior to preparation. A separate complete diet was prepared each day for analysis. All subjects received the same amount of food each day, with the exceptions of bread, cookies, jelly, margarine or butter, and sugar candy, which were allowed ad libitum in order to allow for variation in the caloric intake. These were analyzed separately and the amount consumed was recorded in order to calculate the total daily intakes. Water intakes, a fairly important source of calcium in Lima, were also measured and the calcium content included in the total intake.

The basal diet was similar to the typical prison diet but somewhat more varied and better prepared. Rice was eaten in large amounts twice daily. Potatoes, spaghetti, sweet potatoes, and bread were the other chief foods. Each subject received 100 gm of meat per day and usually a small serving of beans. Soup prepared with a beef bone was eaten for each lunch. Carrots, squash, beets, onions, tomatoes, and other such vegetables were used in moderate amounts. Some fruit, excluding citrus fruits, was allowed twice a day. Coffee or tea was served with every meal. Green vegetables and milk or milk products were completely excluded from the diet except when milk was added as a supplement. This diet was therefore similar to the prison diet but carotene and ascorbic acid were supplied in larger amounts.

The experiment was divided into 5 periods. The first three days on the basal diet were allowed for adjustment to the diet and to gain experience with respect to the amounts of food

to be prepared. Complete urine and fecal collections were then begun. Periods I to III were 10-day periods on the basal diet with none, one cup, and two cups of milk daily, respectively. Period IV was a 6-day period on the basal diet following the highest level of milk intake. During the 5th period of 10 days some of the moderate sources of calcium such as carrots, beans, and the soup bone were removed from the menu and the intake of meat raised to 300 gm per day. This gave a protein intake of approximately 70 gm per day and decreased the calcium intake slightly.

The samples of urine and feces were discarded for a 24-hour period after each change in diet in order to minimize the influence of the previous diet. This is not always sufficient time, as is discussed below, but the use of fecal markers was avoided for fear of losing the cooperation of the subjects. Their cooperation was exceedingly good, although some of the subjects started the experiment convinced that unknown and dangerous procedures would be tried. For this reason, only one blood sample was taken. The total urine, feces, and diet samples were homogenized in a Waring Blendor with distilled water. Aliquots were then combined to give representative samples for the second to 5th day and the 6th to 10th day during each 10-day period. Two balances were thus obtained in subperiods of 4 and 5 days each for 4 diets and one of 4 days in period IV. Samples were discarded for two days after the change from the highest level of calcium to the basal diet. Aliquots of the feces and diet were ashed and taken up in dilute HCl for calcium determination by permanganate titration of the oxalete. The calcium in the urine was precipitated directly. Protein was determined on all samples by the macro-Kjeldahl method using selenium and copper as catalysts.

RESULTS

In figure 1 the total calcium and urinary calcium excretions of each subject have been plotted against the intake. Appropriate regression lines for each set of data have been

calculated and are shown. Only the 7 subperiods on the basal diet (omitting the two periods on the high meat diet) are included in the urinary data. The reason for omitting these will be discussed below. All 9 subperiods are included

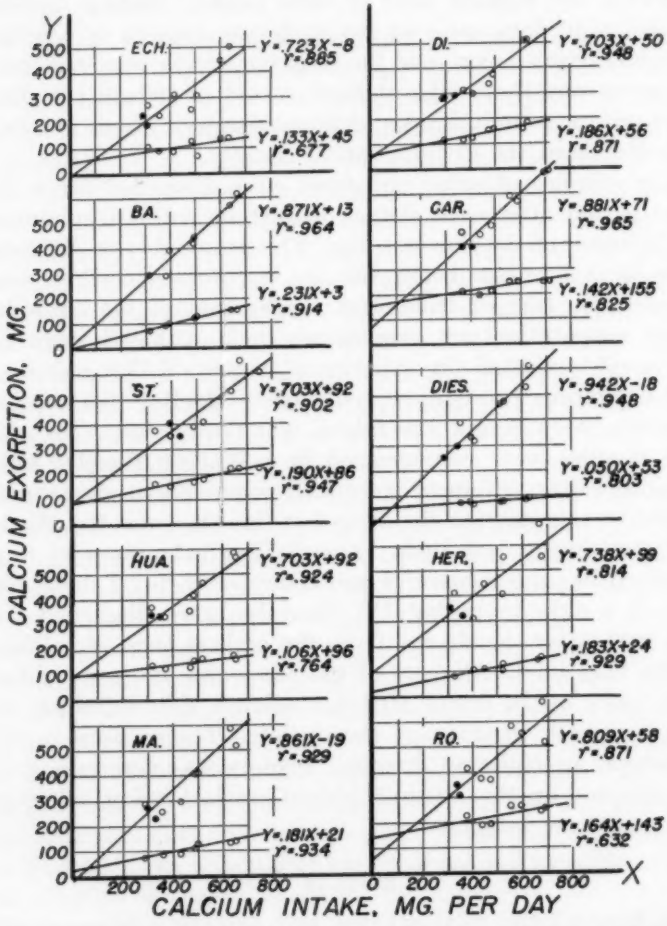

Fig. 1 The relation between calcium intake and output in the various subjects. Lower curves, urinary excretion; upper curves, total excretion. "r" = coefficient of correlation. Open circles, basal diet with or without milk supplements; solid circles, high meat diet (data not included in urinary curves).

in the total excretion (subject Ba. did not participate in the final two) and it is apparent that the data from the high meat diet fall in the expected position and are not markedly different from the values obtained with the basal diet.

The points of interest in the results relating total excretion to intake are: (1) the coefficient of correlation, r, indicating the reliability of the data. The values are high considering the few points in each line; (2) the point at which the regression line crosses the y axis. This would be the predicted excretion at zero calcium intake assuming that the relationship would hold if extended that far. These values in milligrams are given by the constant "a" in the equation $y = bx + a$. In a few instances these are actually negative values but in such cases they are not greatly different from zero. Extension of the line to this extent does not, of course, give reliable values and may not be justified, but we consider the small values obtained worthy of note and discussion; (3) the point at which $y = x$, where excretion equals intake, is the estimated calcium requirement for equilibrium and would prevent calcium depletion. Assuming the body made no further adjustments to the intake, calcium would be lost at levels less than this and stored at higher intakes. These values, indicated for each subject in table 1, varied from 596 mg per day for one subject to negative values for three subjects, which may be considered as zero. The data show 5 subjects with requirements of less than 200 mg per day, 4 with requirements between 300 and 400 mg per day, and one with a requirement of approximately 600 mg per day. This last subject had received numerous calcium salt injections prior to the study, a popular therapy in Lima for colds and other minor illnesses.

The urinary calcium excretion shows in general an excellent correlation with calcium intake. As expected, 4 or 5 urine collections are more accurate than fecal collections in most subjects. Considerable differences may be noted in the magnitude of the urinary calcium and in the slope of the regression lines obtained. The highest urinary values were

obtained with subjects Car., St., and Ro. As has been noted, Car. is known to have received calcium injections, which might easily account for this high excretion. Subjects St. and Ro. were also unique in that they, as well as Ba., were accorded certain prison privileges not received by the other subjects. It is considered likely that a better dietary history and thus better calcium stores could account for these somewhat higher calcium excretion values. The urinary excretion of subject Dies. is also of interest. The regression line has a slope of

TABLE 1

Estimated calcium requirements and average per cent retentions of dietary calcium

SUBJECTS	WEIGHT	HEIGHT	ESTIMATED REQUIREMENT	MEAN RETENTION
	kg	*cm*	*mg/day*	%
Ba.	72	163	101	13
Car.	83	169	596	12
St.	89	178	310	30
Dies.	74	171	0	6
Hua.	61	164	309	30
Ech.	59	161	0	21
Her.	79	161	377	16
Ma.	59	162	0	10
Di.	56	172	168	30
Ro.	65	174	304	19

only 0.05, 5% of the dietary calcium appearing as increased urinary excretion, whereas for all of the other subjects the value was between 10 and 19%. It will be noted also that in this subject the slope of the regression line relating total intake to excretion is very high, at 0.94. This subject retained approximately 6% of the dietary calcium at all the levels of intake.

It may be noted that the estimated total excretion at zero intake is in nearly every case accounted for by the estimated urinary excretion. In several subjects the estimated urinary output at zero intake exceeds somewhat the estimated total

output, but these differences are well within the limits of error of the data.

The combined data for all subjects in all periods have been plotted in figure 2. The line, $y = x$, has also been drawn to indicate the relation of the data to calcium balance. Points falling above the line $y = x$ represent negative balance, points below indicate calcium storage, and the intersection of this line with the true regression line is the estimated average in-

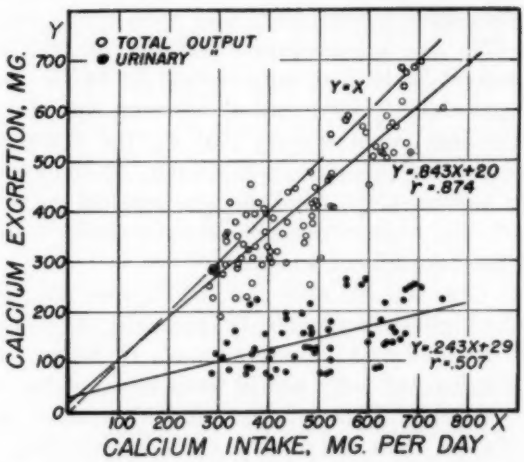

Fig. 2 Relation of calcium intake to output in all subjects. "r" = coefficient of correlation. Open circles, total excretion; solid circles, urinary excretion.

take required to give calcium balance. Clearly, most of the points, regardless of intake, are in the area of positive balance. The mean requirement is estimated to be 126 mg of calcium per person per day. The estimated total excretion at zero intake is estimated as 20 mg per day.

With each change of diet, the urine and fecal samples were discarded for a 24-hour period. It is well known that this may not be sufficient time to prevent completely the influence of the previous diet. In two periods the calcium

of the diet was adjusted upward, and if fecal excretions were delayed an apparent retention of calcium would be obtained. It is apparent that this was true with subject Her. (fig. 1) where considerable differences in excretion were noted in the two periods on the same diet and the higher excretion occurred each time in the second period. There are 12 values in all in which it appears that one might justifiably discard the lower value obtained in the first period. A recalculation was made without these values. Rather suprisingly, the remaining data have a regression line with the equation, $y = 0.905\ x - 0.9$; i.e., an average excretion of 90.5% of the dietary calcium instead of approximately 84.3% (retention 9.5% compared to 15.7%), but the line is tipped to give a zero equilibrium requirement; that is, the regression line does not cross the line $y = x$. Similarly, an omission of the data from subject Car., who appears unusual in this group because of his higher calcium intakes, fails to influence markedly the over-all conclusions.

Subjects Car. and St. were the largest men on experiment and had the highest requirements according to the above calculations. Intakes and excretions were therefore calculated on a per kilogram of body weight basis to see if the variation might be reduced. This relation (fig. 3), treated as in the previous figure, does not improve the correlation. The coefficient of correlation in figure 3 is + 0.869, compared to + 0.874 in figure 2. As suggested by Mitchell and Curzon ('39) and Steggerda and Mitchell ('46), this should not be taken to mean that body size has no effect on calcium requirements, but that individual variations due to other causes are more important than differences in body size. The estimated requirement in figure 3 is higher than that obtained from the data shown in figure 2. Whereas 126 mg per day or 1.8 mg/kg (average weight, 69.9 kg) was obtained by the first method of calculation, the value required to maintain equilibrium is 3.25 mg per kilogram per day in figure 3, and the estimated excretion at zero intake is 0.91 mg/kg/day. These figures may be compared with those obtained by Mitch-

ell and Curzon ('39), which were 3.1 mg/kg at zero intake and 9.75 mg/kg at equilibrium. It is of some interest that the slopes of the regression lines in these two studies are similar and that approximately three times the minimum excretion is required to attain equilibrium in both instances.

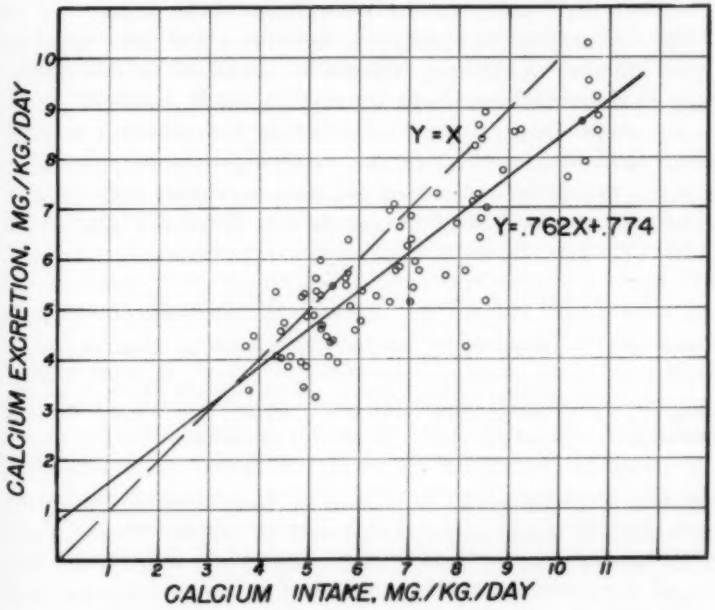

Fig. 3 Relation of calcium intake to output in all subjects expressed on a unit weight basis.

The curves are nearly parallel but ours lie at a lower plane.

It was suggested above that the urinary excretion of calcium was influenced by the high meat diet. The effect of this diet may be shown by comparing the two periods on the basal diet when the intake was comparable. This is done in table 2. It will be observed that, whereas the total average balance was only improved 10 mg, from 19 to 29, by the meat diet,

there was considerable shift in the distribution in feces and urine. Fecal excretion was less and urinary excretion higher on the meat diet. Changes of this magnitude in balance do not alter the over-all relation shown in figure 2 to any great extent, but with the larger values for urinary excretion the regression lines in figure 1 would be markedly changed by these two high values at the lower limit of the data.

The calculation of calcium balance has not been used in these studies. A calcium balance is obtained by the difference in two large numbers to yield a small number. Relatively small errors in the total intake or excretion became large percentage errors in the resulting balance. The percentage utilization of added calcium, however, may be calculated by the method of Steggerda and Mitchell ('41), which

TABLE 2

The effect of added meat on the calcium metabolism

DIET	INTAKE	TOTAL OUTPUT	BALANCE	FECAL EXCRETION		URINARY EXCRETION	
	mg/day	*mg/day*	*mg/day*	*mg/day*	*% of total*	*mg/day*	*% of total*
Basal	362	343	+ 19	214	62	129	38
Plus meat	338	309	+ 29	165	54	144	46

consists of dividing the increment in calcium retention by the increment in intake at any two levels of intake. These values have been shown in table 1 and range from 6 to 30%. One might expect from the literature that those subjects with the lower requirements, i.e., lower calcium stores, might also show the most efficient utilization. This was not found to be true.

Blood samples were obtained during the last two days of experiment. The measurement of hemoglobin, hematocrit, total serum proteins and calcium revealed measurements within the normal range.

DISCUSSION

Minimum calcium requirements may be estimated in several ways from the data presented. One may average the

individual requirements estimated in figure 1, which range from 596 to 0 mg per day. The mean requirement is 216 mg per day. Calculations from combining the data in figures 2 and 3 give values of 126 and 227 mg per day (3.25 mg × 69.9 kg). Each of these values is so low as to indicate that calcium deficiency could scarcely be produced in these adult men on their usual diets. While we have not attempted in this study to obtain minimum intakes by severe and unusual restriction of the diet, it is clear that it would have been difficult to obtain diets of natural foods containing less than the requirements estimated here.

In regions of soft water supplies, lower intakes would result. The water used in Lima was found to contain approximately 70 mg of calcium per liter. Water taken as drinking water supplied up to 104 mg of calcium per day. The total contribution was undoubtedly greater, however, since the same water was used in cooking.

One subject of this group appeared to have the relatively high requirement of about 600 mg per day. It is important to note that this subject must have had rather high calcium stores due to calcium salt injections, and this individual difference may therefore be fairly attributed to past calcium intakes rather than to an idiosyncrasy in calcium metabolism. Estimated requirements of 300 mg in two subjects may also be related to higher calcium intakes. In most of the subjects calcium excretion appeared to be essentially a linear function of intake, decreasing to essentially zero at zero intake.

The increased absorption of calcium observed on the high meat diet was to be expected on the basis of earlier work (Pittman and Kunerth, '39; Kunerth and Pittman, '39; McCance and Widdowson, '42c); retention of calcium was not improved. That it was not, and the fact that the retentions on the basal diet were not unusually high, may be taken as partial evidence that the subjects were not in great need of additional calcium. The extensive review of Knapp ('47) fails to find an adequate explanation for the effect of protein in

increasing urinary calcium excretion, although in some cases this may be related to an increase in the acidity of the diet. This author rejects the belief of McCance and Widdowson ('42b) that urinary calcium is well correlated with calcium absorption and finds a better correlation with total calcium intake. Endogenous factors, presumably endocrine, were suggested as the cause of individual differences in urinary calcium excretion but the prior diet or calcium reserve was not considered as an influential factor.

In an excellent review article Holmes ('44–'45) concludes, principally on the basis of the data of Kinsman et al. ('39), that children probably have no maintenance requirement for calcium and that the calcium intake need not exceed that necessary to supply the calcium required for growth. Ellis and Mitchell ('33) also believe that in young animals calcium ions may be retained at such a rapid rate when intakes are low that the threshold of the kidneys and intestine may never be reached. Considerable data are available from animal studies and some on human beings to indicate that excretion of calcium certainly becomes very low and retention high after considerable periods on low calcium diets. Holmes' review as well as Mitchell's ('44) article on adaptation to undernutrition consider these data.

The present findings lead us to believe that adult men, like children, probably have a very small maintenance requirement for calcium. The evidence is strong that calcium excretion approached zero in the majority of these subjects at very low levels of intake. Certainly one would not expect depletion of the present calcium stores of these individuals on any diet they would be likely to receive.

Past work on quantitative needs for calcium has been reviewed several times (Leitch, '36–'37; Mitchell and Curzon, '39; Steggerda and Mitchell, '46; Sherman, '47; Stearns, '51) and is too extensive to be reviewed in detail here. Perhaps the best-known figure is that obtained in 1920 by Sherman of 0.45 gm per day for the 70-kg adult (6.4 mg/kg). More recent estimates are more nearly equal to 10 mg per kilogram per

day and some are higher (McCance and Widdowson, '42a; Roberts et al., '48). The mean estimated requirement of 0.7 gm per day or 10 mg per kilogram obtained by Steggerda and Mitchell ('46) has a coefficient of variation of 23% or approximately 0.15 gm per day. Ninety-nine per cent of the subjects from such a group should have requirements near 0.7 ± 0.3 gm, or from 0.4 to 1.0 gm per day. This is presumably one justification (see also Macleod and Sherman, '51) for the recent increase in the recommended allowances to 1 gm, from the earlier figure of 0.8 gm (Food and Nutrition Board, '48).

No description of the past dietary habits of the subjects used in the above studies is available but it is likely that they were generally well fed by current standards and had good calcium reserves. Less extensive but convincing data show that calcium equilibrium is attainable at much lower levels of intake (Nicholls and Nimalasuriya, '39; Basu et al., '39; Owen and Irving, '40; Potgeiter, '40) if the subjects have not received high calcium diets. Equilibrium was obtained at levels of from 2 to 4 mg of calcium per kilogram of body weight per day. These values approximate those obtained in this study. While the average stature of people in the tropics is less than that of North Americans and Europeans, Nicholls and Nimalasuriya ('39) estimate that the calcium content per unit of body weight is similar. The average size of our subjects was normal by U. S. standards. In his article on adaptation to under-nutrition, Mitchell ('44) logically relates low calcium excretions and low calcium requirements to smaller calcium reserves in the body. He points out, however, that evidence is lacking to answer the important question as to whether maximum calcium stores are desirable. At present there appears to be no evidence available to indicate the extent to which calcium reserves must be depleted in order to cause conservation of calcium of the order seen in these studies.

It may be questioned whether the studies mentioned above and others might not be more properly considered primarily

as estimates of past dietary habits of the subjects, rather than studies of calcium requirements in the real sense. It is well understood by all workers in the field that studies on protein requirements are simply estimates of the immediately preceding protein intake, unless the subjects are allowed to become adjusted to a low protein diet. That is, equilibrium may be obtained at any level of protein intake above the actual maintenance requirement. Logically the same principles should apply in studies on calcium requirements. That they have not been so applied is undoubtedly due to the time which would be involved. Nitrogen equilibrium at low levels of intake is obtained in a few days. Certainly much longer periods are required to deplete calcium stores. In the studies of Steggerda and Mitchell ('46) some subjects were kept on low intakes for as long as 250 days, during which time calcium losses were more or less constant. Few studies will equal or better this period, but it is not a large portion of adult life and it may not be long in terms of calcium loss. For practical reasons it therefore appears that data on minimum requirements can only be obtained with subjects with low calcium reserves. Clearly, further studies on the relation of these stores to rates of depletion and utilization and their effect on health are needed.

In this regard the extensive work of Sherman and his associates (Sherman and Campbell, '35; Campbell, Pearson and Sherman, '43; Sherman, '47) is important. Rats were shown to be significantly benefitted by levels of calcium in the diet higher than those necessary for reasonable health. However, from the point of view of adult calcium requirements it is not clear from these studies whether the adult animal is benefitted from high intakes if the intake is adequate during pregnancy, lactation, and growth, since the animals were fed the same diet during growth and during the adult periods. Requirements are known to be high during growth, and the adverse effect of the lower calcium diet can be more logically assigned to effects during this period than during adulthood. Kane and McCay ('47) have found calcium re-

quirements of rats to be relatively high during old age, and McCay ('49) comments on the fact that elderly human subjects apparently retain calcium to a greater degree than does the rat. The well-known differences in calcium utilization between rats and human beings (see Holmes, '44–'45) may make the former a poor species for calcium studies. The nutrition of the aged is an important area for investigation which will undoubtedly receive considerable study and may pose its own special problems.

In the final analysis nutritional standards should be related to health. Calcium deficiency probably occurs and could be easily explained, during pregnancy, lactation and growth this type of deficiency need not be discussed here. Little evidence can be found that adult man, in contradistinction to adult women, suffers from calcium deficiency or is benefitted by large calcium stores. Osteomalacia is reported as being widespread in the Orient but Maxwell ('35) says only that, "Naturally men suffer less than women." All of the cases studied by Hannon et al. ('34) and Liu et al. ('35), were women who had gone through several pregnancies, except for one 18-year-old girl. These cases responded readily to vitamin D but not to high levels of calcium. This would therefore appear to have been primarily a vitamin D deficiency rather than a calcium deficiency. Snapper ('50) states that primary calcium deficiency is extremely rare and that osteomalacia responds to vitamin D but not to calcium. Albright and Reifenstein ('48) state that they "are cognizant of no single case of osteomalacia in the United States due to simple lack of vitamin D" and one may presume that this statement may be extended to include calcium deficiency. Osteoporosis, which is widespread in the United States, is defined as a disease of tissue metabolism rather than of calcium metabolism and the same authors "do not believe that calcium lack per se causes osteoporosis" and suggest that "some of the osteopathies which have been attributed to lack of calcium and phosphorus in the diet are really due to protein starvation." Post-meno-

pausal and senile osteoporosis respond to hormone therapy but not to calcium and phosphorus. High protein diets are also recommended.

In conclusion, it would appear that the literature supports the results of this study, which indicate that normal adult man has very low calcium requirements. While we suspect that some readers will be inclined to dismiss these results as a demonstration of adaptation to low calcium diets, we do not believe this justified with respect to our subjects. One with very low requirements was of North European stock. The low excretions obtained are considered simply the normal metabolic adjustment to somewhat reduced calcium stores. It is a general principle that the body is relatively wasteful of nutrients in large supply and conserves those in short supply more efficiently. Adaptation in this sense must be clearly differentiated from the more extensive changes which may be involved in the acclimatization of a species to a new environment, such as the natural selection of the best-fitted individuals. While it is not to be doubted that this may happen to a population in a new nutritional environment, there is little evidence that this was a dominant factor in these studies. Perhaps the phrase "physiological regulation" (Adolph, '43) has a connotation suggesting less permanent changes than "adaptation" and may therefore be perferred in describing these adjustments in metabolism.

SUMMARY AND CONCLUSIONS

1. It is emphasized that very critical examination of dietary standards in terms of costs and benefits is necessary in countries with short food supplies. Unrealistic recommendations are dangerous and serve no useful purpose.

2. Calcium excretions were studied in 10 adult male volunteers at several levels of calcium intake. These men were apparently healthy but had consumed diets of fairly low calcium content over long periods of time.

3. The average estimated calcium requirement to maintain balance in these men was between 100 and 200 mg per

day, depending on the method of calculation. Evidence is presented that the maintenance requirement is related to calcium reserves.

4. These results are discussed in the light of prior estimates of calcium requirement. It is believed that all estimates of calcium requirements represent primarily a study of the previous dietary calcium intake.

5. A review of the literature reveals no solid evidence that calcium deficiency occurs in adult males.

6. The minimum calcium requirement of adult males is probably so low that deficiency is unlikely on most natural diets. When calcium supplies are limited, little effort should be made to increase the consumption of calcium by men. Available calcium should be reserved for children and women in the childbearing age.

ACKNOWLEDGMENTS

The authors are indebted to Señorita Aida Galarreta and chemists Eduardo Viñas, Enrique Alvistur, Renan Urquieta, and Juan Vasquez for much technical assistance in preparing diets and samples and in the chemical analysis.

We also wish to acknowledge the excellent cooperation obtained from the 10 volunteers who actually made the study possible. Special thanks are due to Subject Car. for his invaluable help in carrying out the study.

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STUDIES ON THE INTERRELATIONSHIP BETWEEN METHIONINE AND VITAMIN B_6 [1]

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Levels of methionine only slightly above normal requirements appear to depress growth (Kade and Sheperd, '48; Van Pilsum and Berg, '50), while higher levels are markedly deleterious (Roth et al., '50). This is particularly evident when the diet is low in vitamin B_6 (Sarma et al., '47; Cerecedo et al., '48), and many of the adverse effects of protein in vitamin B_6 deficiency (Miller and Baumann, '45; Cerecedo and Foy, '45) now appear to be due to the methionine therein (Sarma et al., '47; Cerecedo and DeRenzo, '50). Recently Linkswiler et al. ('51) added only 0.4% of methionine to an assay diet containing 18% of casein and observed that the symptoms of vitamin B_6 deficiency were aggravated sufficiently so that a rat assay could be conducted without any preliminary depletion period, graded doses of the vitamin producing progressive increments of growth. The present study deals with the antagonism between vitamin B_6 and methionine, and with certain effects on this relationship of related amino acids.

EXPERIMENTAL

Weanling, male, albino rats from the Sprague-Dawley and Holtzman-Rolfsmeyer animal farms were housed in single,

[1] Published with the approval of the Director of the Wisconsin Agricultural Experimental Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

screen-bottom cages and given food and water ad libitum. The basal diet had the following percentage composition: vitamin-free casein 18; sucrose 73, corn oil 5, salts [2] 4. The following vitamins were added, in milligrams per kilogram of ration: thiamine 2.0, riboflavin 3.0, nicotinic acid 25, calcium pantothenate 20, menadione 10, inositol 100, biotin 0.1, folic acid 2.0, and choline chloride, 1,000. Fat-soluble vitamins were supplied by administration of a 1:10 mixture of halibut liver oil in corn oil to which α-tocopherol had been added. The following levels were given by dropper per rat per week: vitamin A 240 I.U., vitamin D 3.4 I.U., and vitamin E 10 mg. Variations in this diet involved the addition of methionine or other amino acids and the addition of vitamin B_6. The experiments were performed in series consisting of 10 to 12 groups of 5 each; all rats were fed for 4 weeks.

Urine for analysis was collected under toluene for 48 hours. The rats were removed from the collecting funnels at the time of feeding to prevent contamination of the urine with the ration. Animals were fed three times daily for a period of one-half to one hour, after preliminary studies had indicated a normal food consumption on this regimen. Since small amounts of urine were lost under those conditions, the "daily" values for methionine excretion are low, although comparisons between groups are valid. Methionine was determined in the urine with *Leuconostoc citrovorum* 8081 as the assay organism (Steele et al., '49). Vitamin B_6 was determined with *Saccharomyces carlsbergensis* 4228 according to the method of Atkin et al. ('43) as modified by Rabinowitz and Snell ('47).

Pyridoxine vs. methionine

In the absence of vitamin B_6, growth was depressed when as little as 0.4% of methionine was added to the 0.6% of methionine in the casein of the diet (table 1). Pyridoxine progressively counteracted the effects of moderate amounts of

[2] Wesson.

methionine, but when the level of added methionine was 2.5% or more, growth was poor even in the presence of very large amounts of the vitamin. An increase of the level of all other vitamins by a factor of 5 did not restore growth. The ability of pyridoxine to counteract the effects of methionine was observed in many experiments, including one with weanling females and another with young adult males. In the latter, 2.5% of methionine caused a drastic weight loss (50 gm in 4 weeks) in the absence of pyridoxine, but weight was maintained when the diet contained 6 μg of pyridoxine per gram.

TABLE 1

Growth of rats fed various levels of DL-*methionine and pyridoxine*

(Average grams gained in 4 weeks, initial weights 42 to 45 gm [ave.], 5 rats/group.)

DL-METHIONINE ADDED TO DIET[1]	PYRIDOXINE (MG PER KG DIET)			
	0	0.6	6.0	30
%	*gm*	*gm*	*gm*	*gm*
None	34	78	121	..
0.4	17	85	131	..
1.0	5	25	114	..
2.5	..	3	59	54
4.0	..	..	14	11

[1] The basal diet contained 0.6% of methionine in the casein.

Weight was also maintained for 4 weeks on a vitamin B_6-deficient diet free from added methionine, in confirmation of the findings of Miller and Baumann ('44).

In general, the food consumption of all groups was similar in the early stages of the experiments, although food intake tended to parallel body weight after the first week (De Bey, '51). Diets which depressed growth also tended to aggravate acrodynia (swelling, redness, scaliness of paws and scaliness of tail) except when the level of methionine was so high that the rats failed to grow at all. The amount of pyridoxine necessary to prevent acrodynia varied with the amount of methionine in the diet; 0.6 μg per gram was adequate for the lower

levels of methionine but not when 1% of methionine was added to the diet.

Methionine and related compounds in diets deficient in vitamin B_6

Both D- and L-methionine suppressed growth in the absence of vitamin B_6 and appeared to be equal to DL-methionine in this respect (table 2). DL-homocystine also consistently suppressed growth; in two of 5 experiments it appeared to be equal to methionine (table 2, series 3 and 8), while in three others the depressive effect of homocystine was somewhat less than that of methionine (series 5).

Since methionine and homocystine are interconvertible in the body to a considerable extent (Du Vigneaud et al., '39; Bennett et al., '43), an attempt was made to alter this balance by feeding diets high or low in methyl groups (table 2, series 5). The high methyl diet (5c) contained 0.1% of choline and

TABLE 2

Growth of rats fed various sulfur-containing amino acids in diets deficient in vitamin B_6

(Grams gained in 4 weeks, 5 rats/group.)

AMINO ACID ADDED	SERIES [1] 3	SERIES [1] 5a Methyl acceptor	SERIES [1] 5b Low choline	SERIES [1] 5c High choline	SERIES [1] 8	SERIES [1] 10
%	gm	gm	gm	gm	gm	gm
None	38 ± 12.8	47 ± 5.1	52 ± 3.5	48 ± 9.0		
0.6 DL-methionine	15 ± 8.0	13 ± 4.6	30 ± 7.5	32 ± 6.6	23 ± 6.9	20 ± 7.0
0.6 D-methionine	14 ± 9.5					
0.6 L-methionine	13 ± 8.1					
0.54 DL-homocystine	17 ± 12.0	27 ± 4.1	40 ± 11.5	43 ± 10.8	22 ± 15.7	
0.48 L-cystine	38 ± 9.6					35 ± 8.5
0.96 L-cystine	30 ± 15.1					

[1] The rats in the various series differed in starting weight and previous history. The basal diet in series 3, 8 and 10 contained 0.1% of choline. Numbers after ± are standard deviation; least significant differences, series 3, 10.1 gm; series 5, 31.8 gm; series 8, 15 gm; series 10, 18.1 gm.

0.2% of betaine and the low choline diet (5b) contained 0.025% of choline, while the methyl-acceptor diet (5a) was low in choline (0.025%) and contained sufficient nicotinamide (0.24% and 0.71%) to absorb one-half of the methyl groups contained in the methionine of the casein and all of those in the added methionine. These variations in methyl content did not affect the growth of rats not receiving additional methionine, 47 to 53 gm gain in 4 weeks (table 2), but they did affect the response to methionine or homocystine, growth on the methyl-acceptor diet (group 5a) being significantly less than when choline was merely limiting (group 5b) or was present in excess (group 5c). The severity of acrodynia was approximately the same whether methionine or homocystine was fed.

L-cystine, equivalent to 0.6% of methionine (0.48% cystine), did not suppress growth below that in the control groups; growth was less on 0.96% of cystine but the difference was not statistically significant.

Addition of non-sulfur amino acids

Certain amino acids, such as glycine and alanine, have been reported to counteract the adverse effects of high levels of methionine (Roth et al., '50). In the present study glycine, serine, alanine, cystine, and threonine were fed at the levels indicated in table 3. None of these amino acids diminished the growth-inhibiting effect of methionine, with the possible exception of glycine in a diet containing 0.6% of methionine (table 3), and the effects were relatively small as compared with those obtained with vitamin B_6. The lower level of methionine (0.4%), as previously employed (Linkswiler et al., '51), therefore appears to be satisfactory for use in a bioassay. Further evidence of the relative inactivity of the other amino acids under these conditions is the observation that high levels of casein (36% of the diet) depressed growth to the same extent as the methionine it contained (1.2%). The other amino acids in the casein did, however, appear to stimu-

late growth to a level higher than that obtained on 18% of casein, when the diet contained 1 mg of pyridoxine per kilogram (DeBey, '51).

Forms of vitamin B_6

In purified diets the three forms of vitamin B_6 are not equal in promoting the growth of rats unless some modification is made in the ration (e.g., addition of aureomycin; Linkswiler et al., '51) or in the method of administration of the vitamin (injection or feeding separately from the rest of the

TABLE 3

Effect of certain amino acids in combatting growth depression brought about by methionine

(Grams gained in 4 weeks, initial weights 43 to 44 gm [ave.], 5 rats per group.)

AMINO ACIDS ADDED	PYRIDOXINE (MG PER KG DIET)		
	0	0.3	0.6
	gm	*gm*	*gm*
0.4% DL-methionine	18	48	99
0.4% DL-methionine + 1.2% glycine	23	40	95
0.4% DL-methionine + 1.2% DL-serine	18	43	78
0.4 DL-methionine + 1.2% DL-alanine	20	41	95
0.6% DL-methionine	15	..	..
0.6% DL-methionine + 1.8% glycine	25	..	..

diet; Sarma et al., '46). In the present study pyridoxine, pyridoxal, and pyridoxamine were fed at 0.6, 1.2 and 6.0 μg per gram of diets containing 0.4 or 1.0% of added DL-methionine. The lower level of pyridoxine was chosen to promote about half-optimum growth on diets not containing any additional methionine and the medial level for approximately maximum growth, while the high level is 5 times this amount. Additional groups received 20 μg per kilogram of vitamin B_{12}, or 25 μg per gram of aureomycin or penicillin, or a combination of vitamin B_{12} and the antibiotics. As in previous experiments, growth was better with pyridoxine than with the other forms of vitamin B_6 when the level of methionine in the diet was low (0.4%, table 4). When 1% of methionine

TABLE 4

Growth of rats fed various forms of vitamin B_6

(Grams gained in 4 weeks, initial weights 41 to 44 gm, 5 rats per group.)

ADDITIONS TO BASAL DIET	0.6 MG/KG [1]			1.2 MG/KG [1]			6.0 MG/KG [1]		
	Pyridoxine	Pyridoxal	Pyridoxamine	Pyridoxine	Pyridoxal	Pyridoxamine	Pyridoxine	Pyridoxal	Pyridoxamine
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
0.4% Methionine	99[a] [2]	72[a]	80[a]						
1.0% Methionine	26[b]	50[b]	40[b]	101[d]	97[d]	98[d]	127[b]	113[b]	118[b]
				86[c]	88[c]				
1.0% Methionine + vitamin B_{12}	33[a]	27[a]	30[a]	60[d]	96[d]	100[d]			
				72[c]	79[c]				
1.0% Methionine + vitamin B_{12} + aureomycin	29[b]	33[b]	32[b]	91[d]	112[d]				
		49[a]		90[c]	87[c]				
1.0% Methionine + vitamin B_{12} + penicillin				93[d]	102[d]				

[1] All amounts of vitamin B_6 refer to the monohydrochloride. For pyridoxamine dihydrochloride the amounts were 0.7, 1.4 and 7.0 mg/kg.

[2] Letters after the weights refer to series; only the weights within a given series are directly comparable.

was added to the diet, growth with pyridoxine was no better than that with pyridoxal or pyridoxamine. Under many of the conditions imposed, the three forms of the vitamin were approximately equal in activity (table 4), while in others growth with pyridoxal was significantly greater than that with pyridoxine; e.g., when 0.6 μg per gram of the vitamin was added per kilogram of ration containing 1% of methionine, or when higher levels of pyridoxine and pyridoxal were fed in diets containing vitamin B_{12}. This superiority of pyridoxal persisted in the presence of penicillin, and in one of two experiments in which aureomycin was fed. The addition of 20 μg of vitamin B_{12} per kilogram of ration appeared to suppress growth in certain of these experiments (table 4, series 7 and 10, 1% methionine, 1.2 μg per gram of pyridoxine or pyridoxal) and the further addition of aureomycin or penicillin increased growth to rates near or above those obtained on the original diets.

Methionine excretion

Young adult males averaging 200 gm in weight were fed various levels of pyridoxine and methionine for periods of up to 4 weeks (table 5). As expected, the amounts of free methionine in the urine varied with the level of methionine in the diet. In addition, on intermediate levels of dietary methionine (0.6% added) the amount in the urine also depended somewhat upon the level of vitamin B_6 in the diet, deficient rats excreting an average of 750 μg of free methionine daily as compared with 540 μg excreted by the group receiving an adequate level of pyridoxine. This decrease was observed in spite of an increased consumption of food and therefore of methionine. Hence when the results were expressed as percentages of ingested methionine appearing in the urine, the effect of vitamin B_6 in suppressing methionine excretion was particularly evident. The vitamin did not, however, affect the relatively great amounts of methionine in the urine of rats fed 2.5% of the amino acid.

Vitamin B_6 in blood and liver

The amounts of vitamin B_6 in the livers and plasma of these animals varied with the level in the ration, but methionine did not appear to have any effect upon the concentration of vitamin B_6 in either liver or blood (DeBey, '51). When 25 mg of methionine were injected per 100 gm of body weight, the rates of decrease in the methione content of the blood were essentially similar whether the diet was devoid of vitamin B_6 or contained 1 μg per gram.

TABLE 5

Methionine excretion by rats fed various levels of pyridoxine

DL-METHIONINE	PYRIDOXINE	WT. GAINS	METHIONINE CONSUMED	METHIONINE EXCRETED		MG METHIONINE EXCRETED/GM CONSUMED	
				Ave.	Range	Ave.	Range
%	*mg/kg*	*gm/4 wk.*	*mg/day*		*μg/rat/day*		
0	0	— 1	58	130	54–240	2.0	1.2–28
0	1.0	41	83	35	16–45	0.50	0.21–0.66
0	6.0	72	72	27	17–39	0.43	0.23–0.72
0.6	0	0	106	750	670–780	6.8	6.0–7.8
0.6	1.0	56	150	710	410–1,000	4.5	3.2–7.9
0.6	6.0	90	175	540	520–560	2.8	1.8–3.4
2.5	0	— 59	170	1,500	1,100–2,100	9.2	8.2–9.8
2.5	1.0	— 30	210	1,900	950–2,700	8.8	6.8–10.5
2.5	6.0	3	300	2,800	2,400–3,200	11.6	9.4–14.6

DISCUSSION

The fact that methionine intensifies the effects of vitamin B_6 deficiency suggests that either the amino acid itself or one of its metabolites is harmful to the deficient rat. However, the levels of methionine in the liver were not altered in vitamin B_6 deficiency, nor did vitamin B_6 increase the rate of removal of methionine from the blood. Important steps in the reaction

methionine ⟶ homocysteine ⟶ cystathionine ⟶ cystine + homoserine

are catalyzed by pyridoxal phosphate (Binkley and Christensen, '51; Braunshtein and Goryachenkova, '50; Kallio, '51),

and hence any of these compounds might accumulate in the deficient animal, or be degraded to abnormal metabolites. In the present experiments homocystine consistently suppressed growth, although not always as effectively as methionine itself. This latter result, however, may have been misleading, since homocystine is a larger molecule and less soluble than methionine, and may not have been absorbed as well. Furthermore the D-isomer may be less available than D-methionine. Of the metabolites of homocystine, cystine itself was not particularly harmful to the deficient rat; hence experiments with cystathionine or homoserine should prove of interest. Another possibility is that the harmful metabolite of methionine may still contain the methyl group.

The good growth of rats fed pyridoxal or pyridoxamine in diets high in methionine adds another illustration to those in which these forms of vitamin B_6 are at least equal to pyridoxine with respect to producing growth responses in higher animals. Only in purified rations high in soluble carbohydrates is their activity markedly less than that of pyridoxine. It is thus possible that they equal pyridoxine in availability to the body under practical conditions of nutrition. The point is one of some importance in setting probable human requirements for vitamin B_6, since most of this vitamin ingested by man is in the form of pyridoxal or pyridoxamine (Rabinowitz and Snell, '48).

SUMMARY

1. Levels of methionine only slightly above those necessary for growth depressed the growth of rats fed limiting amounts of vitamin B_6. Vitamin B_6 counteracted the effects of moderate amounts of methionine. When the diet contained 2.5% of methionine, high levels of the vitamin failed to restore growth. D-, L- and DL-methionine and DL-homocystine were approximately equivalent in depressing growth; cystine did not depress growth.

2. Acrodynia was aggravated by moderate amounts of methionine (D-, L- or DL-isomers), and by homocystine.

3. Glycine, alanine, serine, cystine, threonine, and additional B vitamins did not affect growth when moderate levels of methionine were added to diets limiting or deficient in vitamin B_6.

4. When the ration contained 1.0% of added methionine, the three forms of vitamin B_6 were approximately equal in promoting growth; in some experiments the most active form of the vitamin appeared to be pyridoxal.

5. The consumption of pyridoxine decreased the excretion of free methionine in the urine of rats fed moderate amounts of the amino acid. Dietary methionine did not affect the concentration of vitamin B_6 in the blood or liver of rats.

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RETENTION OF CRYSTALLINE VITAMIN B_{12} BY HEALTHY MALE INDIVIDUALS FOLLOWING INTRAMUSCULAR INJECTION [1]

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(Received for publication October 3, 1951)

In the preceding paper in this series (Yamamoto, Barrows, Lang and Chow, '51) it was reported that stock colony rats which were maintained on diets providing enough vitamin B_{12} (from milk proteins, 6.5%, and whole liver, 1%) to be apparently adequate for their growth requirements could retain significant amounts of the vitamin following subcutaneous injection. It is, therefore, of interest to ascertain whether healthy human subjects with liberal intakes of diets containing vitamin B_{12} from natural sources (meat, milk) can likewise retain significant quantities following intramuscular administration. To this end, a group of healthy male subjects were given varying doses of vitamin B_{12} by injection. The urinary outputs preceding and following the injection were collected quantitatively. The amount of vitamin B_{12} retained was estimated by computing the difference between the injected dose and the determined activity in the urine samples. The results of this study are reported here.

MATERIALS AND METHODS

Vitamin B_{12}

A sterile solution of crystalline vitamin B_{12} was employed.[2] Its concentration, determined by microbiological assay shortly

[1] The authors wish to acknowledge a grant-in-aid from Sharp and Dohme, Inc., and the National Vitamin Foundation.

[2] It was kindly supplied by Merck and Company.

before this experiment, was 100 μg per milliliter. For the injections either this solution or one prepared from it by dilution with an equal volume of sterile saline solution was used.

Plan of experiment

Twenty healthy male subjects of from 14 to 45 years of age were included in this study. They were divided, at random, into 5 groups. One group, receiving no injection of vitamin B_{12}, served as controls. (Some individuals were used in this group and in one other.) The urinary excretion of vitamin B_{12} by individuals in this group provided basal excretion values. In addition, urine specimens from several individuals in the other groups were collected 24 hours prior to injection; these pre-injection samples were also used to establish basal excretion levels.

Individuals, according to the group to which they were assigned, received single intramuscular injections of vitamin B_{12} solution in amounts equivalent to 20, 30, 50, or 75 μg. These dosages were chosen on the basis of data from previous experiments (Conley, Krevans, Chow, Barrows and Lang, '51) which suggested the possible retention of approximately 20 to 40 μg of vitamin B_{12} by healthy humans. After the injection, which took place during the early part of the morning, the subjects were encouraged to increase fluid intake by drinking coffee. Urine samples were collected from each subject for 24 hours after injection, each in two fractions representing the first 8 hours and the following 16 hours. The volumes of the 8-hour samples ranged from 225 to 1,800 ml, averaging 650 ml. The volumes of the second specimens varied from 455 to 2,370 ml, with an average of 945 ml. All samples were assayed microbiologically for vitamin B_{12} activity, using the method of Skeggs, Nepple, Valentik, Huff, and Wright ('50).

RESULTS

The results of the experiment are given in table 1. It can be seen that the random distribution resulted in relatively

TABLE 1

The retention of vitamin B_{12} by healthy subjects after intramuscular injection

GROUPS	B_{12} INJECTED	NO. OF SUBJECTS	AGE		BODY WT.		MICROBIOLOGICAL ACTIVITY IN URINE [1]				MICROGRAMS B_{12} RETAINED	
			Range	Ave.	Range	Ave.	− 48 hr.	− 24 hr.	+ 8 hr.	+ 24 hr.	Found	Calculated [2]
	μg											
A	0.0	5	14–45	31	150–180	160	< 0.15	< 0.15	..	..	...	...
B	20	5	27–42	33	120–165	145	...	< 0.15 (2)[3]	1.3	< 0.15	18.7 ± 0.2	17.3
C	30	5	27–31	29	155–200	168	...	< 0.15 (1)[3]	1.7	< 0.2	28.3 ± 0.9	24.8
D	50	5	27–34	29	135–180	158	...	< 0.1 (1)[3]	9.8	< 0.2	40.2 ± 5.1	39.0
E	75	4	26–29	28	155–180	174	...	< 0.1 (2)[3]	19.6	< 0.2	55.4 ± 2.7	56.0

[1] Microbiological activity is expressed as equivalent of crystalline vitamin B_{12} in micrograms.

[2] Number in parentheses indicates the number of individuals in the group who participated in the collection of urine during the pre-injection period.

[3] Compare Chesterman, Cuthbertson and Pegler ('51).

comparable average age and body weights in the several groups. (We do not believe that in these experiments variations in age or weight were significant.) The basal excretion of the subjects in group A was consistently less than 150 mμg of vitamin B_{12} in 24 hours or, on the average, less than 50 mμg during an 8-hour period. Values of the same order of magnitude were also obtained from the few individuals whose urinary specimens were collected during the 24 hours preceding the injection. Since the total activity found during the basal period represents a negligible amount in comparison with even the smallest injected dose (20 μg) it was not considered in calculating the quantity of B_{12} retained by the body.

Paper chromatographs were run on all of the 8-hour post-injection samples. In each instance spots with virtually the same Rf value as that associated with vitamin B_{12} in the same system were observed.

Several points of interest are worth noting. In the first place, regardless of the amount of vitamin injected the microbiological activity in the urine specimens reached a maximum within 8 hours after injection. Only a negligible amount was present in the specimens collected during the following 16 hours. In the second place, subjects in group B receiving 20 μg of vitamin B_{12} intramuscularly excreted approximately 1 μg of vitamin B_{12} activity, indicating a retention of as much as 19 μg. With an injected dosage of 30 μg (group C), the microbiological activity in the urine of these subjects remained at substantially the same level; thus, the amount of vitamin B_{12} retained by this group of subjects was as much as 29 μg. With a further increase in the dosage, to 50 μg (group D) and to 75 μg (group E), the urinary excretion of microbiological activity was substantially elevated, with resultant net average retentions of 40 and 55 μg of B_{12}, respectively.

These data, therefore, demonstrate that substantial amounts of vitamin B_{12} can be retained by healthy human males following intramuscular administration to individuals whose dietary

history indicates presumably adequate oral intake of this vitamin. The amount of vitamin B_{12} retained appears to be dependent on or related to the amount injected. An empirical equation describing the relationship between administered dosages and excretion was suggested by Chesterman, Cuthbertson and Pegler ('51) as being $E = D - 1.2D^{0.89}$, where E = amount vitamin B_{12} excreted and D = amount injected. Within the presently reported range of administered dosages, our data are in quite good agreement with this equation, as is shown in the last column of table 1.

DISCUSSION

Berk et al. ('48) demonstrated that oral administration of vitamin B_{12} in small doses failed to bring about therapeutic response in patients with pernicious anemia, unless gastric juice from healthy individuals was also administered at the same time. If vitamin B_{12} is required by the normal human, and it is difficult to imagine otherwise, the daily requirement is probably adequately met in healthy individuals by diets which contain components, such as milk or meat products, rich in vitamin B_{12}. This conclusion is also substantiated by the small frequency with which reported clinical deficiency of this vitamin (pernicious anemia) is encountered.[3] In view of our previous findings that orally administered vitamin B_{12}, even in massive doses of the crystalline material or as naturally occurring complexes in meat or milk products, is poorly absorbed in normal subjects, or in normal laboratory animals (Chow, Barrows and Lang, '50; Chow, Rosenblum, Silber, Woodbury, Yamamoto and Lang, '51), it is conceivable that healthy subjects are not "saturated" with vitamin B_{12}. This hypothesis is in agreement with the findings reported in this communication which demonstrate the retention of sub-

[3] We recognize that the pernicious anemia syndrome can be regarded as the result of intrinsic factor insufficiency and is, perhaps, more accurately described in those terms than as vitamin B_{12} deficiency.

stantial quantities, roughly proportional to the administered parenteral dose.

Calculation of the amount retained was made by taking the difference between the administered dosage and the amount found in the urine. This implicitly assumes that administered vitamin B_{12} is not excreted in an altered form or as a split product not available to the test organisms used for microbiological assay. The reasonableness of these assumptions is supported not only by our paper chromatography studies but by the data reported elsewhere (Yamamoto, Barrows, Lang and Chow, '51; Chow, Rosenblum, Silber, Woodbury, Yamamoto and Lang, '51) on the distribution coefficient of the excreted vitamin following administration to rats of vitamin B_{12} tagged with radioactive Co^{60}. Their validity is further strengthened by the finding that certain tissues, such as the muscle, liver, kidneys, and so forth, of rats injected subcutaneously with the radioactive vitamin contain large amounts of Co^{60} which, in some cases, has been demonstrated to be explicitly associated with vitamin B_{12} (Rosenblum, Chow, Condon and Yamamoto, '52). These considerations therefore led us to the conclusion that the difference between the injected and the excreted vitamin represents the amount retained by the tissues.

Although our procedure for the measurement of retention is similar to a "saturation test" (Kline and Eheart, '44) for vitamin C, which is considered valuable as a means of determining adequacy of ascorbic acid intake, we have no data at the present time to indicate that the retention values reported here for vitamin B_{12} represent the physiological requirements of healthy humans. Nevertheless, our results demonstrate the ability of healthy males to retain large quantities of vitamin B_{12} following a single, intramuscular administration; amounts presumably many times larger than those estimated to be present in normal diets. The normal subjects used in these experiments were not saturated with vitamin B_{12} in spite of a presumably adequate intake in food.

SUMMARY

Crystalline vitamin B_{12} was administered intramuscularly to groups of healthy male subjects at the following levels: 20, 30, 50, and 75 μg. Urinary collections from each subject were made during 24 hours after injection, each in two fractions representing the first 8 hours and the following 16 hours. The vitamin B_{12} microbiological activities in the urine specimens were measured. These data permitted calculation of the amount retained, by taking the difference between the injected and the excreted vitamin. It was found that more than 50 μg of vitamin were retained daily by these subjects, depending on the dosage administered.

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THE EXCRETION OF N^1-METHYLNICOTINAMIDE AND CREATININE AS INFLUENCED BY VITAMIN B_{12} AND VARIOUS METHYL DONORS [1]

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(Received for publication July 27, 1951)

In the presence of vitamin B_{12} the requirement of labile methyl groups necessary for the growth of chicks (Schaefer et al., '49b; Gillis and Norris, '49; Briggs et al., '50) and for the protection of rats against hemorrhagic kidneys (Schaefer et al., '49a) and fatty livers (Drill and McCormick, '49; György and Rose, '50) is reduced. Shive ('50) and Davis and Mingioli ('50) have described an interrelationship between methionine and vitamin B_{12} in bacterial metabolism. These facts suggest that vitamin B_{12} plays an important role in some aspect of the formation or transfer of methyl groups.

The original concept of transmethylation was based on the classical experiments by duVigneaud and his associates, who demonstrated the synthesis of methionine from homocystine if certain methyl groups were provided preformed in the diet of the rat (duVigneaud, '48). Bennett et al. ('44), however, found that under their experimental conditions animals continued to grow on diets containing homocystine as the sulfur-containing amino acid in the absence of any known source of labile methyl groups. Subsequent research, reviewed by duVigneaud et al. ('50a) and Bennett ('50), has led to our present knowledge that in the presence of vitamin B_{12} and homocystine the growth of animals is possible on diets deficient in known labile methyl group donors (Bennett,

[1] Paper 2685, Scientific Journal Series, Minnesota Agricultural Experiment Station.

'50; Jukes et al., '50; Stekol and Weiss, '50). Evidence that methionine was actually synthesized in these experiments has thus far rested largely on observed increments in weight which presumably involved the synthesis of protein and hence of methionine.

It is generally believed that the methyl groups required for the methylation of nicotinamide and guanidoacetic acid, forming N^1-methylnicotinamide and creatine respectively, are derived directly from methionine (Borsook and Dubnoff, '47). This concept was used as the basis of the present study, in which the excretion of the methylated derivatives of nicotinamide and guanidoacetic acid was used as an indication of the synthesis of methionine by rats receiving a labile-methyl-free diet supplemented with various combinations of homocystine, choline, betaine, formate, and vitamin B_{12}.

EXPERIMENTAL

The rats used in this study were offspring of mothers maintained from weaning on ration S–7, composed of the following ingredients, expressed in grams per kilogram: expeller type soybean oil meal, 722.4; hydrogenated vegetable fat,[2] 70; sucrose, 152; salt mix (Schultze, '50), 40; corn oil, 10; DL-methionine, 5.6. Vitamin supplements, which included 0.1% choline chloride and biotin, were added at levels previously described in work from this laboratory (Schultze, '50). A high incidence of mortality was observed in the F_1 generation, especially during the two-week post-weaning period. The administration of vitamin B_{12} not only improved weight gains during this period but also reduced the incidence of mortality to zero. Poor growth and high mortality during the early post-weaning period have also been described by Borson et al. ('50) with their vitamin B_{12}-deficient diet. The young were fed ration S–7 until they were transferred to the amino acid ration described below. They were therefore severely deficient in vitamin B_{12} at that time.

[2] Crisco.

In any one experiment litter mates surviving the two-week post-weaning period were divided in two groups, each containing two males and two females (55 to 65 gm), and fed ad libitum a basal ration containing, in grams per kilogram, the following ingredients: amino acid mixture[3] (Stekol and Weiss, '50), 176.5; hydrogenated vegetable fat,[4] 100; sucrose, 150; salt mix (Schultze, '50), 40; agar, 20; corn oil, 10; dextrinized cornstarch, 502.5; L-cystine, 1.0. Vitamin supplements (Schultze, '50) from which choline had been omitted were added in the usual manner. In the experiment involving the administration of nicotinamide this vitamin was also omitted from the basal ration. Within each experiment the basal ration was modified to include one of the following supplements added at the expense of dextrin: 0.8% DL-methionine; 1.0% choline chloride; 0.6% DL-homocystine; 0.6% DL-homocystine plus 0.8% choline chloride, 0.8% betaine chloride, or 0.4% sodium formate. One group of rats was injected intraperitoneally with 0.5 μg vitamin B_{12}[5] three times per week; the other group served as uninjected controls.

At the beginning of the experiment the animals were transferred to individual metabolism cages, and the urine was collected for a three-day period in beakers containing 1 ml of toluene and 1 ml of glacial acetic acid. At the end of the first three-day collection period, the rats in one series of experiments were given an intraperitoneal injection of 20 mg of nicotinamide, and in the second series an intraperitoneal dose of 20 mg of guanidoacetic acid[6] neutralized to pH 7.0. The urine was collected for three additional days and the experiment was terminated on the 7th day. The animals were weighed daily and the amount of food consumed daily by each animal was recorded. To make allowances for differences in food consumption, weight changes were calculated

[3] The amino acids used were commercial products not subjected to further purification.

[4] See footnote 2, page 224.

[5] One-tenth per cent trituration of crystalline vitamin B_{12}, Merck and Co., Inc., Rahway, N. J.

[6] H. M. Chemical Co., Ltd., Los Angeles, Cal.

as grams gain in weight per gram of dietary N ingested over a period of 7 days.

The urine and wash water were diluted to 100 ml and kept at 5°C. until used for analysis. Suitable aliquots of the filtered urine samples were analyzed for N^1-methylnicotinamide by the method of Huff and Perlzweig ('47) or for creatinine [7] by a modification of the procedure of Clark and Thompson ('49). Using the original procedure described by the latter authors, it was found that sugars leached from spilled food by the urine and wash water seriously interfered with the determination of creatinine when the urine was autoclaved directly with the picrate buffer. This interference was avoided by autoclaving the urine with an equal volume of 0.1 *M* KCl-HCl buffer (pH 2.0) and then treating the autoclaved urine with the picrate buffer at room temperature.[8] The N^1-methylnicotinamide or creatinine content of the urine samples collected subsequent to the administration of nicotinamide or guanidoacetic acid, respectively, was corrected for the excretion of these methylated derivatives during the period preceding injection. The methyl groups excreted in the form of N^1-methylnicotinamide or creatinine were calculated on a molar basis.

RESULTS

The results obtained in these short-term experiments are summarized in tables 1 and 2. The responses of different groups to various dietary and other treatments were evaluated by the "t" test (Snedecor, '46) and the principal comparisons are shown in table 3.

Growth

With the exception of the two groups receiving DL-homocystine, the weight changes of the corresponding groups of

[7] "Creatinine" as used here refers to the total creatinine value obtained after autoclaving the urine in the presence of acid. This includes the preformed creatinine plus the creatine converted to creatinine.

[8] This procedure permitted a quantitative determination of creatinine in urine in the presence of glucose added in concentrations as high as 250 mg %. Reducing sugars as glucose in urine specimens never exceeded 50 mg %.

TABLE 1

Weight change of rats receiving an amino acid ration with various supplements in the presence or absence of vitamin B_{12}

SUPPLEMENT TO BASAL AMINO ACID RATION	VITAMIN B_{12}	NICOTINAMIDE INJECTION (20 MG)			GUANIDOACETIC ACID INJECTION (20 MG)		
		Gain in weight	N consumed	Weight change [2]	Gain in weight	N consumed	Weight change [2]
	$\mu g/wk.$[1]	*gm/rat/wk.*	*gm/rat/wk.*	*gm/gm N*	*gm/rat/wk.*	*gm/rat/wk.*	*gm/gm N*
None	0	— 11.5	0.70	— 16.5 ± 0.67	— 12.0	0.65	— 18.5 ± 1.54
None	1.5	— 10.0	0.67	— 15.0 ± 1.18	— 13.0	0.74	— 17.6 ± 0.61
0.8% DL-methionine	0	+ 14.5	1.31	+ 11.1 ± 0.71	+ 13.5	1.23	+ 11.0 ± 0.52
0.8% DL-methionine	1.5	+ 17.5	1.27	+ 13.8 ± 0.47	+ 18.0	1.21	+ 14.9 ± 0.66
1.0% choline chloride	0	— 9.5	0.64	— 14.8 ± 0.68	— 10.0	0.61	— 17.2 ± 0.71
1.0% choline chloride	1.5	— 8.0	0.63	— 12.7 ± 0.67	— 9.0	0.59	— 15.1 ± 1.32
0.6% DL-homocystine	0	+ 4.0	1.11	+ 3.6 ± 1.15	— 1.0	0.91	— 1.1 ± 1.52
0.6% DL-homocystine	1.5	+ 16.5	1.32	+ 12.5 ± 0.76	+ 13.0	1.04	+ 12.5 ± 0.44
0.6% DL-homocystine + 0.8% choline chloride	0	+ 7.0	0.81	+ 8.6 ± 0.40	+ 10.0	1.14	+ 8.8 ± 0.59
0.6% DL-homocystine + 0.8% choline chloride	1.5	+ 11.5	0.90	+ 12.8 ± 1.11	+ 15.0	1.26	+ 11.9 ± 0.56
0.6% DL-homocystine + 0.8% betaine chloride	0	+ 9.5	1.16	+ 8.2 ± 0.23			
0.6% DL-homocystine + 0.8% betaine chloride	1.5	+ 11.5	1.06	+ 10.9 ± 0.58			
0.6% DL-homocystine + 0.4% sodium formate	0	+ 3.0	1.20	+ 2.5 ± 0.50			
0.6% DL-homocystine + 0.4% sodium formate	1.5	+ 14.0	1.15	+ 12.2 ± 0.90			

[1] Vitamin B_{12} in the amount of 0.5 μg administered by intraperitoneal injection three times per week.
[2] Mean ± standard error of the mean, based on 4 animals per group.

TABLE 2

Excretion of methyl groups as N¹-methylnicotinamide or creatinine by rats receiving an amino acid ration with various supplements in the presence or absence of vitamin B_{12}

SUPPLEMENT TO BASAL AMINO ACID RATION	VITAMIN B_{12}	URINARY N¹-METHYLNICOTINAMIDE AS ($-CH_3$)			URINARY CREATININE AS ($-CH_3$)		
		Basal [2]	Test [3]	Test-basal [4]	Basal [2]	Test [5]	Test-basal [4]
	μg/wk.[1]	$mM \times 10^{-2}$/rat	$mM \times 10^{-2}$/rat	$mM \times 10^{-2}$/rat	$mM \times 10^{-2}$/rat	$mM \times 10^{-2}$/rat	$mM \times 10^{-2}$/rat
None	0	21.6	36.8	15.2 ± 0.72	113.3	114.6	1.3 ± 0.27
None	1.5	19.7	35.8	16.1 ± 0.60	99.7	101.8	2.1 ± 0.49
0.8% DL-methionine	0	43.9	109.9	66.0 ± 2.76	74.2	93.8	19.6 ± 1.24
0.8% DL-methionine	1.5	46.5	118.0	71.5 ± 1.85	81.6	102.6	21.0 ± 1.43
1.0% choline chloride	0	22.0	41.6	19.6 ± 1.23	108.5	128.7	20.2 ± 1.14
1.0% choline chloride	1.5	23.8	47.2	23.4 ± 2.52	98.3	119.6	21.3 ± 1.02
0.6% DL-homocystine	0	23.3	41.8	18.5 ± 1.03	69.1	72.8	3.7 ± 0.62
0.6% DL-homocystine	1.5	23.8	67.7	43.9 ± 2.29	70.6	90.4	19.8 ± 1.92
0.6% DL-homocystine + 0.8% choline chloride	0	35.4	80.9	45.5 ± 2.13	74.5	92.7	18.2 ± 1.93
0.6% DL-homocystine + 0.8% choline chloride	1.5	33.0	90.8	51.8 ± 1.70	74.9	94.5	19.6 ± 2.02
0.6% DL-homocystine + 0.8% betaine chloride	0	39.8	74.8	35.0 ± 1.59			
0.6% DL-homocystine + 0.8% betaine chloride	1.5	45.0	113.2	68.2 ± 3.57			
0.6% DL-homocystine + 0.4% sodium formate	0	25.2	42.2	17.0 ± 0.77			
0.6% DL-homocystine + 0.4% sodium formate	1.5	31.7	82.7	51.0 ± 3.24			

[1] Vitamin B_{12} in the amount of 0.5 μg administered by intraperitoneal injection three times per week.
[2] Seventy-two-hour excretion preceding injection of nicotinamide or guanidoacetic acid.
[3] Seventy-two-hour excretion following an intraperitoneal injection of 20 mg of nicotinamide.
[4] Mean ± standard error of the mean, based on 4 animals per group.
[5] Seventy-two-hour excretion following an intraperitoneal injection of 20 mg of guanidoacetic acid.

rats injected with nicotinamide or guanidoacetic acid, respectively, were very similar. Rats receiving the unsupplemented basal ration lost weight and the administration of either choline or vitamin B_{12} or of both did not prevent this. The feeding of homocystine, however, prevented the marked loss in weight of the rats injected with nicotinamide and greatly

TABLE 3

Significance of group comparisons[1]

SUPPLEMENTS TO BASAL AMINO ACID DIETS OF 2 GROUPS COMPARED		"t" VALUES			
		Nicotinamide injected		Guanidoacetic acid injected	
Group 1	Group 2	Wt. change	N^1-MNA	Wt. change	Creatinine
B_{12}	None	1.099	0.966	0.543	1.420
M	H + CH	2.954	5.867	2.787	0.566
M	H + B	3.895	10.972	....	....
M + B_{12}	M	3.021	1.655	4.614	0.740
M + B_{12}	H + B_{12}	1.080	9.398	2.904	0.502
CH	None	1.785	3.102	2.421	16.178
CH + B_{12}	None	4.017	3.132	1.282	19.120
CH + B_{12}	CH	2.205	1.353	1.386	0.721
CH + B_{12}	B_{12}	1.691	2.818	0.442	16.934
H	None	15.055	2.620	8.056	5.533
H	H + F	0.931	1.164	....	....
H + F	None	26.600	1.715	....	....
H + B_{12}	H	6.444	10.011	8.527	7.648
H + B_{12}	H + CH	4.563	0.514	4.810	0.588
H + B_{12}	M *	1.348	6.177	2.287	0.087
H + B_{12}	H + F + B_{12}*	0.256	1.789	....	....
H + B	H	3.905	8.687	....	....
H + CH	H	4.090	11.367	6.078	7.149
H + CH	H + B	0.873	3.932	....	....
H + CH + B_{12}	H + CH	3.570	4.538	3.813	0.501
H + B + B_{12}	H + B	4.309	8.267	....	....
H + CH + B_{12}	H + B + B_{12}*	1.518	2.560	....	....
H + F + B_{12}	H + F	10.410	10.200	....	....

[1] Weight change or excretion of methylated metabolites was greater in group 1, except in the three cases marked *, where group 2 excreted more N^1-MNA. Abbreviations: B_{12} = vitamin B_{12}; M = DL-methionine; CH = choline chloride; F = sodium formate; B = betaine chloride; H = DL-homocystine; N^1-MNA = N^1-methylnicotinamide. Probability of differences being due to chance: at 5% probability "t" = 2.447; at 1% probability "t" = 3.707.

decreased that of the rats injected with guanidoacetic acid.[9] Homocystine in the presence of choline, betaine, or vitamin B_{12} produced a growth response which was comparable to that produced by the methionine-supplemented ration. The administration of vitamin B_{12} effected a further improvement in the growth of rats receiving diets containing methionine or homocystine plus choline or betaine.

Excretion of N^1-methylnicotinamide

The excretion of N^1-methylnicotinamide when choline or homocystine was added to the diet of rats receiving no vitamin B_{12} was increased to a small but significant degree. When homocystine plus vitamin B_{12} or homocystine together with choline or betaine were administered, the excretion of N^1-methylnicotinamide was greatly increased, whereas formate was without effect in this respect. Vitamin B_{12}-injected rats on diets containing homocystine plus choline or betaine excreted more N^1-methylnicotinamide than their uninjected litter mates. The highest, and nearly equal, excretion of N^1-methylnicotinamide was observed when rats received methionine with or without vitamin B_{12} or homocystine plus betaine plus vitamin B_{12}.

The basal excretion of N^1-methylnicotinamide can be observed to be likewise dependent on the nature of the methyl donor, and, in most instances, paralleled changes in the net excretion of this metabolite discussed above. By injecting nicotinamide an amplification of these differences is obtained.

Excretion of creatinine

Additions of betaine and formate to the basal ration were not included in this series of experiments. When the basal ration was supplemented with choline, methionine, or homocystine plus choline, the excretion of creatinine was markedly

[9] Paper chromatography of the homocystine used in these experiments revealed the presence of methionine. Analysis of the homocystine by the method of McCarthy and Sullivan ('41) showed methionine to be present to the extent of 3.1%.

enhanced. Simultaneous administration of vitamin B_{12} with these supplements elicited no further response. When the basal diet, however, was supplemented with homocystine alone, the simultaneous injection of vitamin B_{12} increased the excretion of creatinine to a level similar to that observed when the diet contained methionine or choline.

It is of interest to note that the basal excretion of creatinine was somewhat greater on those diets on which the animals suffered losses in weight; namely, the unsupplemented basal ration and the basal ration supplemented with choline. Tidwell ('46) had previously observed that increased creatine excretion was associated with the weight loss of rats on diets deficient in labile methyl groups.

DISCUSSION

It has been demonstrated (Perlzweig et al., '43; Cantoni, '51) that the methyl groups required for the *in vitro* formation of N^1-methylnicotinamide from its unmethylated precursor, nicotinamide, must be derived from methionine or homocystine plus betaine. *In vivo* evidence of the role of methionine as an obligatory methyl donor in this reaction was offered by Handler and Dann ('42). Foà et al. ('45), however, found that by controlling the food intake of their animals they were able to demonstrate that choline could serve as a direct methyl donor for the methylation of nicotinamide. In the experiments reported here choline was relatively ineffective as a methyl donor for the formation of N^1-methylnicotinamide; hence the increase in the excretion of the latter when homocystine was fed in conjunction with choline, betaine, or vitamin B_{12} can be ascribed to a preliminary synthesis of methionine.

Borsook and Dubnoff ('40, '45) found that the conversion of guanidoacetic acid to creatine by liver slices was enhanced only in the presence of methionine or homocystine plus choline. *In vivo* evidence as to the specific methyl donor for creatine synthesis is somewhat conflicting. DuVigneaud ('41) has referred to experiments using deuterocholine and deutero-

methionine which indicate that methionine is far more effective than choline as a methyl donor for the formation of creatine in the rat. Stetten and Gail ('42), however, found that the inclusion of guanidoacetic acid in the diet of rats markedly reduced the choline content of the liver without impairing growth. This was interpreted by Handler and Dann ('42) as indicating a direct utilization of the methyl groups of choline for the synthesis of creatine. The ability of choline to serve as a methyl donor for the *in vivo* synthesis of creatinine to at least the same degree as methionine is indicated by the present study. The enhanced excretion of creatinine in the presence of homocystine plus choline, therefore, *cannot* be accepted as evidence of the synthesis of methionine. The observation, however, that vitamin B_{12}-injected rats receiving the basal diet supplemented with homocystine excreted far more creatinine than their uninjected litter mates strongly suggests the synthesis of methionine.

The precise manner in which vitamin B_{12} participates in the biosynthesis of methionine is still obscure. Since glycine and serine may function as precursors of labile methyl groups via formate (duVigneaud et al., '50b; Jonsson and Mosher, '50; Sakami and Welch, '50; Arnstein, '51; Berg, '51; Mitoma and Greenberg, '51), Stekol and Weiss ('50) have suggested that vitamin B_{12} functions in the *de novo* synthesis of methyl groups which become available for methionine synthesis. It has recently been reported (Arnstein and Neuberger, '51; Stekol et al., '51) that vitamin B_{12}-deficient rats utilize glycine for choline synthesis to a much smaller extent than rats receiving vitamin B_{12}. If the role of vitamin B_{12} is to enhance the formation of formate from suitable precursors, it is difficult to explain from the data presented here why formate in the absence of vitamin B_{12} was not effective in the methylation of homocystine for growth and the excretion of N^1-methylnicotinamide. There remains the possibility, however, that vitamin B_{12} may facilitate the conversion of formate to methyl groups which then become available for the methylation of homocystine.

Our data show that vitamin B_{12} in conjunction with homocystine consistently increased the excretion of N^1-methylnicotinamide even in the presence of dietary sources of labile methyl groups. Dinning et al. ('51) have likewise noted that vitamin B_{12} enhances the utilization of betaine or choline for the production of white blood cells in the rat to the same extent as methionine alone. Oginsky ('50) has shown that the liver homogenates from vitamin B_{12}-deficient rats formed less methionine from homocystine and choline or betaine than similar preparations from the livers of rats receiving vitamin B_{12}. Experiments involving the use of methionine, the methyl group of which was labeled with C^{14} and deuterium, have shown that the methyl groups of choline and betaine in the tissues bore the same ratio of C^{14} to deuterium as the ingested methionine (Keller et al., '49). It would appear, therefore, that the effect of vitamin B_{12}, at least in the presence of adequate dietary sources of labile methyl groups, cannot be accounted for by the *de novo* synthesis of labile methyl groups.[10]

The fact that the effect of vitamin B_{12} on N^1-methylnicotinamide excretion was marked even when betaine was substituted for choline would indicate that vitamin B_{12} probably does not function in the conversion of choline to betaine which Dubnoff ('49) and Muntz ('50) have shown to be the active methyl donor to homocystine. A possible explanation for the role of vitamin B_{12} in the biosynthesis of methionine is that it facilitates the *utilization* of methyl groups, perhaps through the mechanism proposed by Dubnoff ('50, '51); that is, by increasing the reduction of homocystine to homocysteine, which is the direct acceptor of methyl groups from suitable donors. This concept is supported by the observation that

[10] While this manuscript was in preparation, Gillis and Norris ('51) reported that vitamin B_{12} increased the growth of chicks receiving a methionine-deficient diet containing homocystine plus choline or betaine. They concluded, however, that since the effect of vitamin B_{12} was strictly additive this could not be accepted as evidence that vitamin B_{12} is concerned with the transfer of preformed methyl groups.

vitamin B_{12}-deficient rats have lower blood sulfhydryl content than litter mates receiving vitamin B_{12} (Ling and Chow, '51).

SUMMARY

Young vitamin B_{12}-deficient rats were fed a labile-methyl-free ration with the following supplements in the presence or absence of vitamin B_{12}: none; methionine; choline; homocystine; homocystine plus choline, betaine, or formate. The urinary excretion of N^1-methylnicotinamide or creatinine was determined following an intraperitoneal dose of nicotinamide or guanidoacetic acid.

Only when the basal ration was supplemented with homocystine in the presence of choline, betaine, or vitamin B_{12} were the growth and the excretion of N^1-methylnicotinamide similar to those observed with the methionine-supplemented ration. The administration of vitamin B_{12} effected a further improvement in growth and increase in N^1-methylnicotinamide excretion when the diet contained homocystine plus choline or betaine. Formate in the absence of vitamin B_{12} was ineffective as a methyl donor to homocystine.

When the basal ration contained homocystine, the administration of vitamin B_{12} or additional supplementation with choline caused an increase in growth and excretion of creatinine comparable to the increase obtained with the methionine-supplemented ration. Choline alone, although incapable of supporting growth, effected a similar increase in creatinine output.

The implication of these results with respect to the role of vitamin B_{12} in the biosynthesis of methionine has been discussed.

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NUTRITION RESURVEY IN BATAAN, PHILIPPINES, 1950 [1]

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THREE FIGURES

(Received for publication August 8, 1951)

INTRODUCTION

Biochemical measurements of the effects of the enriched rice program in Bataan are reported here. The measurements were made in December, 1950, after the population had consumed rice enriched with iron, thiamine and niacin for two years. The previous survey of August, 1948, conducted by Burch et al. ('50), established a base line for thiamine levels and for other nutrients in body fluids before the introduction of enriched rice into Bataan on October 1, 1948. Evidence for nutritional deficiency due to lack of thiamine was found in the low blood and urinary thiamine levels of the population sample examined. Evidence of multiple dietary deficiencies was found in low serum values for vitamin C and carotene and in very low hemoglobin levels.

Earlier clinical studies by Salcedo et al. ('48) had established the high incidence of beriberi and the high mortality rate due to beriberi in Bataan.

After fortified rice had been consumed by the population for some time, a second clinical survey during the period

[1] This study was supported by grants from the Williams-Waterman Fund of the Research Corporation, from the Nutrition Foundation, Inc., and from the U. S. Public Health Service, Office of Research Grants, National Institutes of Health.

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from July 1, 1949, to February 28, 1950, was conducted by Salcedo et al. ('50). The results in 7 municipalities revealed an average decline of 89% in the incidence of beriberi. In Orani, the municipality in which the biochemical tests were done, the 1949–50 clinical survey revealed an incidence of 0.7% of beriberi. No cases of frank beriberi were found. The total per cent reduction in incidence of beriberi in Orani was 93%; i.e., from 9.4% in the first survey to 0.7% in the second. Beriberi mortality in the experimental area declined from 263 per 100,000 in 1947–48 to 28 per 100,000 in 1949–50. Thus both the decrease in the incidence of beriberi and the decline in the mortality rate reflected the beneficial effects of enriched rice.

The method of rice enrichment by means of a rice pre-mix and the system of distribution to mills in Bataan have been described (Salcedo et al., '50). Previous statements regarding the actual level of enrichment, however, are somewhat in error, since they do not take into consideration the fact that an extra allowance of 20% of the nutrients was provided against the contingency of deterioration of the pre-mix during storage in a tropical climate. The pre-mix actually used was such as to provide, in each 1,000 gm of enriched rice, 5.5 mg of thiamine, 40 mg of niacin and 33 mg of iron, assuming no deterioration during storage of the pre-mix or the enriched rice. Actually, the deterioration encountered in storage was less than 10%. Based on a daily consumption of 360 gm of rice per capita, the enriched rice, as purchased, furnished 2.0 mg of thiamine, 14 mg of niacin and 12 mg of iron. These amount to more than the daily allowances of thiamine and niacin and the equivalent of the daily allowance of iron recommended for a moderately active woman by the Food and Nutrition Board of the National Research Council, U.S.A.

GENERAL OBSERVATIONS

Striking improvements had taken place in Orani between 1948 and 1950. A new Health Center of the United Nations

International Children's Emergency Fund occupied a prominent place in the town. Electricity was available from 5:00 o'clock in the afternoon until midnight, whereas none had been available in 1948. The interest and alertness of the people had greatly increased; few were apathetic. Particularly noticeable was the gain in weight and the generally more healthy appearance of the people. Many of the babies who were *in utero* at the time of the 1948 survey accompanied their mothers in 1950, looking well-fed and happy. None had the aphonia previously observed in some infants. Many of the expectant mothers of 1948 were again pregnant in 1950. With few exceptions their general appearance was much improved. Not a single person among those who came for testing showed any visible signs of beriberi.

EXPERIMENTAL

Dietary survey

Dietary histories from the 200 test persons were taken one to three weeks prior to the blood tests. Each person was given a dietary history booklet and instructed in its use. Three-day diets were recorded by each subject on the mid-week days of Tuesday, Wednesday and Thursday. Food eaten was listed after each meal. Some difficulties were encountered in obtaining accurate records from this population group. Many failed to understand the importance of regular listing and left blank spaces. Others neglected to record any kind of measures.

For the compilation of data, one of the three-day diets from each subject was chosen at random and analyzed for calories, minerals and vitamins. For the calculations of intakes, food tables from the Philippine Institute of Nutrition (Bocobo et al., '51), from McLester ('49) and from the U. S. Department of Agriculture (Watt and Merrill, '50) were used. The results of estimations made of the diets of 200 Filipinos in Bataan revealed an average daily intake of 2,200 cal., which includes 132 gm protein, 36 gm fat, 338 gm carbohydrate, 0.3

gm calcium, 0.7 gm phosphorus, 22 mg iron, 8,000 I.U. vitamin A, 1.9 mg thiamine, 45 mg ascorbic acid, 0.3 mg riboflavin, and 24 mg niacin. Because of the methods which were necessarily used in the collection of these data, they represent estimates of the dietary intakes rather than accurate measurements. They furnish, nevertheless, a basis for calling attention to some points of interest concerning the diet of this group. The average caloric intake of the test group was slightly below (about 100 cal.) the requirement recommended by the National Research Council, Philippines, for expectant mothers and moderately active women, and very low (about 600 cal.) compared to the National Research Council requirement for lactating women. The intakes of thiamine, niacin, iron, protein and vitamin A were satisfactory according to the ratings of the National Research Council. Very low intakes of ascorbic acid, calcium and riboflavin were revealed.

Methods of sampling

Analyses for nutrients in blood were made on 200 test persons from Orani and surrounding barrios. Analyses had been done on 64% of these persons in 1948. Within three weeks prior to the chemical tests they were clinically examined for symptoms of beriberi and classified according to the rating previously described by Burch et al. ('50) as frank, suspected, doubtful or non-symptomatic. Only 44 showed mild symptoms which would place them in the doubtful classification. All others were non-symptomatic. No frank or suspected cases were found. In the test persons classed as doubtful in 1950, only the subjective symptoms of tingling, numbness, occasional cramps and muscle tenderness were noted. There were no objective signs of beriberi found on test subjects at this time. The most prominent ones found in 1948 were edema, pallor, paresis, cardiac involvement and impairment of reflexes.

A comparative description of the population samples of 1948 and 1950 is shown in table 1. Groups as similar as pos-

sible to the 1948 groups were obtained, including as many of the same test persons as were available.

Blood was obtained by finger puncture. Hemoglobin samples in ammoniacal solution were stored on ice overnight, and measured at the Philippine Institute of Nutrition in Manila. Blood serum protein was determined at the time of the survey by the gradient tube specific gravity method. Trichloroacetic acid filtrates were prepared from blood samples for duplicate thiamine analyses and from serum for ascorbic acid determinations. These filtrates and the serum samples for vitamin A, carotene and riboflavin were transported on dry ice to the Philippine Institute of Nutrition, where the analyses were done.

TABLE 1

Population samples of 1948 and 1950

YEAR	TOTAL NO. OF PERSONS	NO. OF MALES	NO. OF FEMALES			NO. OF PERSONS IN EACH CLASS OF BERIBERI			
			Total	Pregnant	Lactating	Frank	Suspected	Doubtful	Nonsymptomatic
1948	202	25	177	54	56	20	52	68	50
1950	200	28	172	45	72	0	0	44	156

Methods of analysis

Analyses were made by the same methods as in 1948: serum protein, Lowry and Hunter ('45); calculation of hematocrit, Phillips et al. ('43); ascorbic acid, Lowry et al. ('45); vitamin A and carotene, Bessey et al. ('46); blood thiamine, Burch, Bessey and Lowry ('52). Hemoglobin was determined colorimetrically, Evelyn ('36). In addition, the serum riboflavin was measured fluorometrically, Burch et al. ('48).

Urine samples were preserved with acetic acid in a final concentration of 0.1 N. Urinary thiamine was measured by the same method used in the 1948 survey. Riboflavin was determined by measuring the fluorescence reducible by hydrosulfite in samples pretreated with excess permanganate, fol-

lowed by treatment with peroxide. The thiamine and riboflavin were calculated per gram of creatinine. Analyses were performed on 47 urine samples.

Results of the analyses

Blood and urinary thiamine. Comparison of the data in 1950 and 1948 (table 2) reveals a 20% increase in blood thiamine. Statistically significant increases ($P < 0.01$) are apparent in both the entire test group and in the 127 persons whose tests were repeated in 1950. No differences in the blood levels of persons classed as doubtful and non-symptomatic were observed in either survey. So far as levels of blood thiamine or urinary excretion are concerned, these two groups were not differentiated. This would logically suggest that the mild symptoms described clinically, which placed persons in the doubtful class with respect to beriberi, may have been due to causes other than lack of thiamine.

The per cent of persons whose thiamine levels were below 3 μg per 100 ml of blood decreased from nearly 34 to 4. A concomitant increase from 28 to 66% in those whose levels were above 4.1 μg per 100 ml of blood shows the trend toward satisfactory levels (fig. 1).

For comparison of the improvement in the blood thiamine levels and in the clinical classification of the various groups shown in table 2, the results on persons actually tested both in 1948 and 1950 were averaged (data not shown). Of the frank and suspected group, 44 persons were retested in 1950. Fifteen classed as doubtful and 29 as non-symptomatic in 1950 showed an actual increase in blood thiamine of 0.9 μg per 100 ml, or 28%. Of the doubtful group, 45 persons were retested. Thirteen classed as doubtful and 32 as non-symptomatic showed an increase of 0.8 μg per 100 ml, or 22%. Of the non-symptomatic group, 36 were retested in 1950; all were classed as non-symptomatic, and they showed an increase of 0.7 μg per 100 ml, or 17%. The change in blood thiamine of these smaller groups is essentially the same as that of the

TABLE 2

Thiamine levels in blood, red blood cells and urine of Filipinos in Bataan

DATE	CLASS OF BERIBERI	NO. OF PERSONS	THIAMINE [1]		NO. OF PERSONS	THIAMINE [1]	
			Blood	R.B.C.[2]		Blood	Urine
			μg/100 ml	*μg/100 ml*		*μg/100 ml*	*μg/gm creatinine*
1948	Frank and suspected	72	3.4 ± 0.11	9.0 ± 0.31	20	3.1 ± 0.26	47 ± 3
1950	Frank and suspected	0					
1948	Doubtful	68	3.7 ± 0.12	9.3 ± 0.29			
1950	Doubtful	43	4.2 ± 0.13	10.6 ± 0.45	10	3.7 ± 0.22	239 ± 44
1948	Non-symptomatic [3]	50	4.0 ± 0.18	9.4 ± 0.45	20	3.9 ± 0.27	71 ± 4
1950	Non-symptomatic [3]	152	4.3 ± 0.09	10.0 ± 0.43	31	4.2 ± 0.30	246 ± 40

[1] Results are shown here as the mean ± the standard error of the mean.

[2] The thiamine in red blood cells was calculated from data on thiamine in whole blood and the hematocrit, assuming the plasma contained 0.1 the amount in red cells.

[3] Samples collected daily from one of the authors (H.B.) during the test periods in Bataan, 1948 and 1950, gave average blood thiamine values of 5.9 and 5.7 μg per 100 ml, respectively.

entire group, although the per cent increase in the frank and suspected group is higher. Part of this increase in blood thiamine admittedly results from an increase in the percentage of red cells, since the bulk of the circulating thiamine is contained in these cells. Nonetheless, a 10% increase in thiamine concentration in the red cells was observed.

Large and statistically significant ($P < 0.01$) increases in the urinary excretion of thiamine were apparent (table 2). These increases were calculated on the basis of the amount

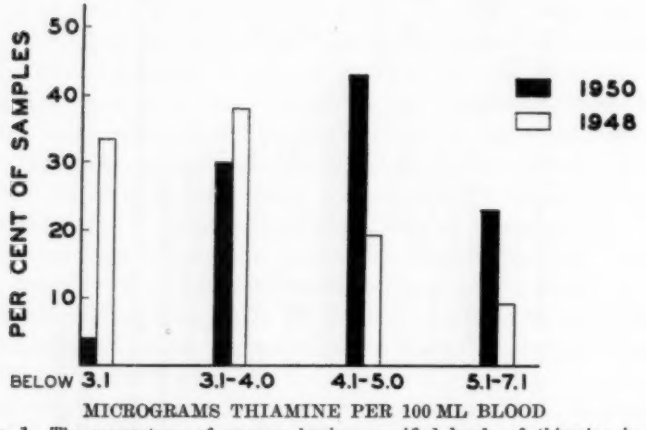

Fig. 1 The percentage of persons having specified levels of thiamine in the blood in 1948 and 1950.

of urinary thiamine excreted per gram of creatinine. A gram of creatinine may be considered roughly the amount excreted per day by a small adult weighing 110 pounds. It is not possible to set an exact figure for excretion of thiamine below which impairment may occur. However, it is probably true that an excretion of less than 50 μg per gram of creatinine is unsatisfactory (Williams et al., '42). In the 1948 Bataan survey, 70% of the persons showing symptoms of beriberi and 45% of the non-symptomatic persons excreted 50 μg or less of thiamine per gram of creatinine. In the 1950 survey

(fig. 2), only 14.5% of those tested excreted less than 50 μg per gram of creatinine, thus demonstrating a remarkable improvement.

Hemoglobin. Large increases in hemoglobin occurred (table 3). The hemoglobin value increased 2.1 gm per 100 ml of blood, a statistically significant change ($P < 0.01$). Similarly, in repeat cases (table 5), a highly significant increase was

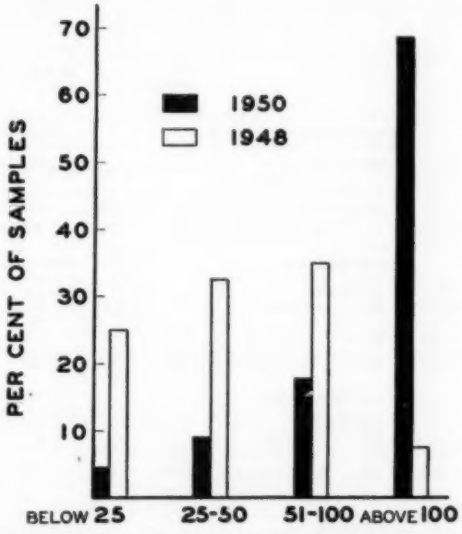

Fig. 2 The percentage of persons having specified levels of thiamine in the urine in 1948 and 1950.

shown. A large swing is apparent (fig. 3) in the distribution of the samples toward a much more satisfactory level in 1950. Even more impressive is the increase in the average value for the group of 37 repeat cases, whose initial hemoglobin was below 10 gm per 100 ml. The 1948 average of 8.7 gm increased to 11.7 gm in 1950, an actual increase of 3 gm over a two-year period. Only three of the 37 failed to show an increase in hemoglobin. Such a marked rise in hemoglobin,

along with the increase in thiamine levels, could scarcely fail to contribute greatly toward the general improvement in health noted among the population sample studied.

The increase could not have been attributed to individual medical treatment, since no report of hemoglobin values or of other nutrients in individual cases was sent to the Philip-

TABLE 8

Chemical findings on the entire test groups of 1948 and 1950

YEAR	NO. OF PERSONS	ANALYSIS			NO. OF PERSONS	ANALYSIS		
		SERUM PROTEIN				HEMOGLOBIN		
		gm/100 ml				*gm/100 ml*		
		< 6.4 %		Mean [1]		< 9.1 %	< 11.1 %	Mean [1]
1948	201	29		6.7 ± 0.07	201	16.5	49.5	10.9 ± 0.14
1950	195	31		6.9 ± 0.11	198	4.5	19.0	13.0 ± 0.12
		SERUM CAROTENE				SERUM VITAMIN A		
		µg/100 ml				*µg/100 ml*		
		< 75 %	< 125 %	Mean		< 20 %	< 30 %	Mean
1948	193	89	97	47 ± 1.3	193	2	11	42 ± 0.6
1950	187	64	88	72 ± 3.4	187	14	43	34 ± 1.1
		SERUM ASCORBIC ACID				BLOOD THIAMINE		
		mg/100 ml				*µg/100 ml*		
		< 0.2 %	< 0.4 %	Mean		< 3 %	< 4 %	Mean
1948	202	14	53	0.46 ± 0.01	190	34	72	3.6 ± 0.07
1950	200	20	54	0.48 ± 0.03	195	4	34	4.3 ± 0.07
		SERUM RIBOFLAVIN				URINARY RIBOFLAVIN		
		µg/100 ml				*µg/gm creatinine*		
		< 2.5 %	< 3.0 %	Mean		< 100 %	< 200 %	Mean
1950	91	11	29	3.7 ± 0.1	47	40	89	120 ± 9

[1] The value following the ± sign is the standard error of the mean.

pines after the 1948 survey. No medical treatment other than the very limited amount already existing prior to the 1948 survey was available in Bataan. Except for the change in the quality of food consumed due to the enrichment of rice, no other major alteration in the diet occurred (Salcedo et al., '50).

Some of the increase in the average hemoglobin might be expected to be due to the somewhat smaller number of pregnant and larger number of lactating women in the 1950 group. However, comparison of the hemoglobin levels found in the pregnant and lactating groups of 1948 and 1950 revealed only a small difference in their hemoglobin response (table 4). A considerably larger response occurred in the women who were not subjected to either stress. This may indicate insufficient iron and possibly other factors associated with hemoglobin formation to meet adequately the demands of pregnancy and lactation.

TABLE 4

Hemoglobin levels of pregnant and lactating women in 1948 and 1950

DATE	NO. PREGNANT	HEMOGLOBIN Mean [1]	NO. LACTATING	HEMOGLOBIN Mean	NO. OTHERS	HEMOGLOBIN Mean
		gm/100 ml		*gm/100 ml*		*gm/100 ml*
1948	54	10.0 ± 0.2	56	11.4 ± 0.1	65	10.8 ± 0.4
1950	45	11.4 ± 0.2	72	12.6 ± 0.2	55	13.0 ± 0.3

[1] The value following the ± sign is the standard error of the mean.

Serum protein. A very small increment was observed in the serum protein averages (table 5). Both the means and the distributions (table 3) remained essentially unchanged and were satisfactory for such a population sample.

Serum vitamin A. The serum vitamin A values decreased in 1950 (table 5), with a rather striking rise in the per cent of samples below 30 μg per 100 ml (table 3). Several factors may have a bearing on this decrease, such as the scarcity of imported oils fortified with vitamin A, a possible seasonal variation in the sources of vitamin A, or poor conversion of carotene into vitamin A. The mean value still falls within a satisfactory range (30 to 70 μg per 100 ml) despite the shift toward a lower level.

Serum carotene. The serum carotene value had increased by 1950 but still remained far below the range adjudged satisfactory (100 to 300 μg per 100 ml).

The increase is attributed to the local harvest of sweet potatoes in December, 1950. The blood serum levels do not reflect sufficient increase to show that the amounts consumed were adequate to make the carotene level satisfactory. The change in the per cent of persons whose carotene levels were below 75 μg per 100 ml of serum was from 89% in 1948 to 64% in 1950.

Serum ascorbic acid. Neither the serum values (table 5) for ascorbic acid nor the general distribution of the samples

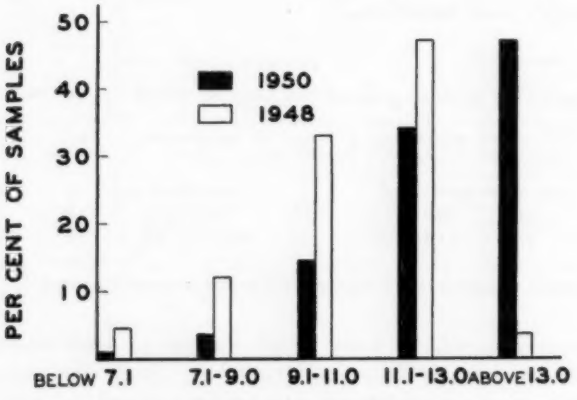

Fig. 3 The percentage of persons having specified levels of hemoglobin in 1948 and 1950.

changed appreciably during the two years (table 3). The concentration of ascorbic acid in the serum for both 1948 and 1950 was low and adjudged unsatisfactory (satisfactory range, 0.7 to 2.0 mg per 100 ml). It is interesting that 72 lactating women in 1950 showed a considerably lower level of serum vitamin C than 45 pregnant women — 0.34 and 0.50 mg per 100 ml, respectively. The depletion apparently represents the normal tendency to maintain a generous supply of ascorbic acid in human milk. Similar differences were found in similar 1948 groups. These results emphasize the demand for vita-

min C during lactation and point toward a need for further improvement in the Filipino diet.

Riboflavin. The 1950 average urinary excretion of 120 μg of riboflavin per gram of creatinine (table 3) represents an undesirably low level, since anything less than 200 μg of riboflavin per gram of creatinine is probably undesirable (Aykroyd et al., '49). Forty per cent of the 47 persons tested fell below 100 μg per gram of creatinine. No riboflavin was added to the enriched rice, which makes any great increase in intake

TABLE 5

Chemical findings on 127 persons examined in both 1948 and 1950

	1948 MEAN [1]	1950 MEAN [1]	DIFFERENCE [2] (1950 minus 1948)
Hemoglobin, *gm/100 ml*	10.9 ± 0.24	12.9 ± 0.18	+ 2.0 ± 0.30
Blood thiamine, *μg/100 ml*	3.5 ± 0.12	4.3 ± 0.08	+ 0.8 ± 0.15
Urinary thiamine, *μg/gm creatinine* [3]	65 ± 13	253 ± 49	+ 188 ± 51
Serum protein, *gm/100 ml*	6.7 ± 0.05	6.8 ± 0.06	+ 0.1 ± 0.08
Serum ascorbic acid, *mg/100 ml*	0.49 ± 0.03	0.48 ± 0.03	— 0.01 ± 0.04
Serum vitamin A, *μg/100 ml* [4]	42.3 ± 1.1	35.0 ± 1.4	— 7.3 ± 1.8
Serum carotene, *μg/100 ml* [4]	48.4 ± 2.6	74.0 ± 4.4	+ 26.6 ± 5.1

[1] The value following the ± sign is the standard error of the mean.
[2] The value following the ± sign is the standard error of the difference.
[3] Twenty-three cases repeated.
[4] One hundred and twenty cases repeated.

unlikely between 1948 and 1950 in such an isolated agricultural population with established dietary habits. These data warrant the assumption that no significant quantities of milk were consumed, and emphasize the need for more adequate riboflavin and calcium sources in the diet. More difficult to interpret is the level of serum riboflavin found. No differences from the levels of normal individuals were observed. These data are in accord with the finding of no drop in the total riboflavin in the serum of infants depleted of riboflavin (Snyderman et al., '49), although the free riboflavin apparently decreased. The red blood cell riboflavin would have been

a more valuable determination, since this shows a fall parallel with depletion, but facilities in Orani did not permit the collection of red cell samples.

SUMMARY

A nutrition resurvey by microchemical methods and a dietary and clinical study have been made in Bataan on 200 persons. Sixty-four per cent of these were examined in both 1948 and 1950.

Results of chemical analyses of blood and urine in 1948 and 1950 are informative concerning the effects of current intake of certain of the vitamins and minerals. The marked increases in blood and urinary thiamine and in hemoglobin indicate a major change in the intake of thiamine and iron in the diet. These increases are mainly attributable to the consumption of enriched rice, since its introduction constituted the only major dietary change in this isolated agricultural peninsula where the pattern of food intake was well established. Indirectly, the finding of undesirably low urinary riboflavin in 1950 contributes to the evidence that the increases in thiamine levels were due to rice enrichment, since riboflavin and thiamine often occur together in foods. The findings point toward the need for the further enrichment of rice by adding riboflavin.

The findings revealed no significant change in the low concentration of vitamin C in serum. This would indicate that consumption of foods rich in vitamin C, such as leafy vegetables, tomatoes and citrus fruits, had not changed. Likewise the serum carotene values remained low, a finding attributable also to the continued low intake of vegetables and fruits The serum vitamin A and protein remained in a satisfactory range.

The results of the dietary study are for the most part in accord with the chemical findings. They also point toward satisfactory intakes of thiamine and iron and toward lack of ascorbic acid and riboflavin. In addition, they reveal a critical lack of calcium.

The chemical findings on changes in thiamine levels and in hemoglobin concur with the clinical reports on the improvement in health of the people following the inauguration of the enriched rice program in Bataan.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. C. G. King, Professor of Chemistry at Columbia University and Scientific Director of the Nutrition Foundation, Inc., and to Dr. R. R. Williams, Chairman of the Williams-Waterman Committee for the Combat of Dietary Diseases, for advice and encouragement in conducting the survey. They also wish to acknowledge the assistance of Mr. Leon G. Alejo, Miss Isabel Concepcion, Miss Josefina Manalo, Miss Victoria Pobre, Mrs. Rosita Salud and Mr. Cesar Yaptinchay with some of the chemical determinations, and to thank Dr. Arsenio Regala and other members of the Institute of Nutrition for kind assistance, and Mr. Emilio Naval, Governor of Bataan, and the people of Orani for their cooperation.

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A PROCEDURE FOR MEASURING THE DIGESTIBILITY OF PASTURE FORAGE UNDER GRAZING CONDITIONS

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ONE FIGURE

(Received for publication August 22, 1951)

Although pasture grass is generally considered to be the most economical of livestock feeds, research dealing with the feeding and economic value of grass in the form grazed by animals has lagged far behind the research conducted on other kinds of feeds. This lag appears to be attributable largely to the lack of a reliable method for estimating digestibility and rate of consumption under grazing conditions.

As a result of studies conducted with sheep and cattle fed various hays, silage, and freshly clipped forage, an indicator method employing a naturally occurring plant chromogen absorbing light at 406 mμ has been proposed for measurement of digestibility and intake of roughages (Reid et al., '50). The use of this method is feasible only under conditions in which the samples of feed obtained for analysis truly represent the feed consumed by the experimental animals. Since this situation exists only in trials in which hand feeding is practiced, a real need for a procedure usable in grazing experiments remained.

As a result of these experiments (Reid et al., '50) and subsequent ones on hays, it was observed that a quantitative relationship existed between the chromogen content of the consumed roughages and that of the feces voided (Woolfolk,

'50). Since animals graze selectively, it is impossible, except perhaps by chance and possibly in the case of a few plant species, to sample manually in such a way that the composition of the hand-chosen sample is representative of the forage consumed by grazing animals. For this reason we have given some attention to characteristics of fecal composition, measurement of which might serve as a substitute for forage sampling. Since the chromogen level of the feces appeared to correspond to that of the ingested grass, it was indicated that this relationship might reflect the nature of the grass actually consumed, thereby amounting to forage sampling. Also, this would eliminate the need for analyzing grass for chromogen, a difficult procedure with some forage samples and one which, when conducted on forage sampled manually, does not represent the actual chromogen content of the *grazed* grass.

It is obvious that should definite mathematical relationships exist between the composition of the feces and that of the *consumed* grass, more accurate estimates of the value of pastures could be made than is possible at the present time. Therefore, the objectives of these experiments were: (a) to examine the relationship between the chromogen content of feces and that of the *consumed* forages; (b) to ascertain the accuracy with which the chromogen content of the forages may be predicted from the chromogen content of the feces; and (c) to outline a procedure for measuring the digestibility of pasture grass under grazing conditions.

EXPERIMENTAL

Since it was desirable to examine forages varying in a number of characteristics which animals would encounter in grazing, conventional digestion trials were conducted in which clipped, whole forages of different botanical composition and of various stages of growth were hand-fed. This procedure allowed a study of forages varying greatly in chemical composition, ratio of legume to non-legume plants, ratio of leaves

to stems, and degree of digestibility. Known quantities of whole forage from each of 4 sources were hand-fed continuously during the grazing season to 4 groups of three steers each. A definite and constant amount of forage was fed daily, beginning 10 to 15 days prior to, and during, each trial. Measurement was made of the total quantity of feces voided during 4 consecutive days at intervals of 15 to 17 days by means of fecal collection bags and harnesses. Since it had been observed in studies of roughages that, when great care was taken during a long preliminary feeding period, the results of a 4-day fecal collection period were comparable to those of a 7- to 10-day period, the shorter period was adopted to minimize the effects of changing forage composition.

The botanical composition of the forage mixtures and the stage of growth of the individual plants fed during each trial are shown in table 1. It was observed that the botanical composition varied considerably from one area to another within the same pasture.

Sequence of trials

Trials 1, 2, 3, and 4 on forage 1 and trials 1, 2, and 3 on each of forages 2 and 3 were conducted at successive stages of maturity at 17-day intervals. Trials 4 and 5 on each of forages 2 and 3 were conducted successively at 17-day intervals on the regrowth of these forages following an earlier clipping. Trial 5A preceded trial 5 on forage 3 by only 5 days. The group of steers used previously in the trials on forage 1 were employed in trial 5A with forage 3. Trials 1, 2, and 3 on forage 4 were conducted successively, with 16- and 15-day periods intervening between trials 1 and 2 and trials 2 and 3, respectively.

Modification of method of measuring chromogen concentration

The procedure described previously for calibrating the method of measuring the chromogen concentration (Reid et

TABLE 1

Botanical composition of mixtures and growth stages of individual plants

FORAGE MIXTURE	TRIAL NUMBER					
	1	2	3	4	5	5A
Forage 1						
Ky. bluegrass	EH[1] (43.5)[2]	EBl (44.8)	S. (40.0)	S. (34.4)		
Timothy	V. (26.6)	EH (31.5)	EBl (40.0)	FBl (30.8)		
Meadow fescue	V. (12.7)	H. (9.4)	FH (14.8)	S. (18.6)		
Quack grass	V. (9.1)	V. (5.0)				
Weeds	(8.0)	(9.4)	(5.2)	(1.6)		
Red top				FBl (14.6)		
Forage 2						
Timothy	V. (38.9)	EH (19.4)	EBl (8.4)	V-EH (44.5)	EH (8.5)	
Quack grass	V. (31.1)	H. (57.9)	S. (86.0)	V. (21.4)	V. (30.7)	
Alfalfa	V. (18.2)	V. (17.3)	V. (5.1)	V. (19.4)	V-EBl (57.8)	
Clover	V. (11.5)	V. (2.8)	V. (0.6)	V-EH (11.3)		
Weeds	(0.3)	(2.6)		(3.5)		
Dead material					(2.9)	
Forage 3						
Quack grass	V. (66.2)	H. (84.3)	S. (52.1)	V. (19.5)	V. (24.7)	V. (43.1)
Timothy	V. (20.7)	EH (9.4)	EBl (39.9)	V-EH (4.8)	EH (2.8)	EH (6.1)
Alfalfa	V. (9.9)		V. (7.3)	V. (64.0)	V-EBl (60.1)	V-EBl (45.8)
Clover	V. (3.1)	V. (3.5)		V. (5.8)	V. (4.4)	V. (0.2)
Ky. bluegrass		EBl (2.8)	S. (0.7)			(0.5)
Weeds	(0.1)				(0.9)	
Dead material				(5.9)	(7.1)	(4.3)
Forage 4						
Timothy	V. (58.0)	EH (70.0)	FBl (78.0)			
Ky. bluegrass	(27.0)	(25.0)	(18.0)			
Quack grass	(6.0)	(2.0)				
Weeds	(9.0)	(3.0)	(4.0)			

[1] The following abbreviations are employed for the growth stage: V = vegetative, EH = early head, H = head, FH = full head, EBl = early bloom, FBl = full bloom, and S = seed.

[2] Figures in parentheses represent percentages of various plants in the mixture. All percentages except those for forage 4 are expressed on dry basis; data for forage 4 are based on green weight.

al., '50) was modified prior to conducting these experiments. As a result of experiments in which rations consisting of concentrates and hay were fed, it was found that complete recovery of the ingested chromogen was not obtained in the feces (Woolfolk, '50). Since different extraction treatments did not remedy this discrepancy, an investigation of the procedure for calibrating the method was made. This investigation revealed that the use of Na_2CrO_4 is not entirely satisfactory for the standardization of the method as originally proposed, because extracts of chromogen do not correspond to the Beer-Lambert law in precisely the same manner as solutions of Na_2CrO_4. Although similar chromogen values were obtained for most substances by both methods of calibration when the recommended sample sizes and dilution rates (Reid et al., '50) were employed, extracts containing very large or very small quantities of chromogen were the ones in which the greatest errors were encountered. Since concentrate feeds contain very small quantities of chromogen absorbing light at 406 mμ, large errors were encountered when the chromogen content was determined in terms of the Na_2CrO_4 standard. As a result of these observations, the standardization procedure was modified as follows:

(a) A concentrated extract of mixed forages, roughages and feces (resulting from the ingestion of a variety of forages and roughages) was prepared, using 85% acetone (by volume) as the solvent.

(b) From the concentrated extract a portion to be used as a standard was diluted sufficiently to obtain a low galvanometer deflection (1 to 10% transmission of light). Chromogen concentration values were assigned to this extract, and each successive subdilution of it based upon a reference concentration of Na_2CrO_4. The amount of light absorbed by a reference solution containing 5.05 mg % Na_2CrO_4 was termed equivalent to that absorbed by an extract containing 10 units of chromogen per 100 ml.

(c) From the measurements made of the amount of light absorbed by the subdilutions of the concentrated extract

expressed on dry basis; data for forage 4 are based on green weight.

(above, under b), a light transmission — chromogen concentration line or equation or both was determined. For the particular instrument employed in these studies, this relationship is expressed by the equation:
$Y = 57.39 - 28.74X$, where: Y = concentration of chromogen in units per 100 ml of extract, and X = log of the per cent of transmitted light.

The extraction procedure reported previously (Reid et al., '50) was modified early in these experiments. Since it was found that alternate blending and cooling of the macerate eventually caused the blendor cups to leak, and since it was observed that this practice was not advantageous, the sample to be extracted was blended continuously for 5 to 7 minutes without cooling. Aside from this modification, the procedure for preparing the extract was the same as that described earlier (Reid et al., '50).

RESULTS AND DISCUSSION

In order to obtain maximum accuracy in relating the chromogen content of feces to that of the consumed forage, the concentration of chromogen in the consumed forage was computed from data on the quantity of feces voided, the chromogen concentration of the feces and the quantity of forage consumed. This procedure was employed because the chromogen analysis of feces is made rather easily and accurately, whereas that of forages (particularly at late stages of growth) is sometimes difficult to execute. This computation assumes that the data obtained in the conventional digestion trial are accurate and that chromogen is completely indigestible, as reported previously (Reid et al., '50).

However, it is recognized that complete recovery of ingested chromogen from the feces is not essential in the computation of digestibility, provided that the rate of recovery is consistent for all forages or that the chromogen concentration of the feces is related to the digestibility of the forage. During the course of these experiments it was observed that the chromogen concentration of the feces was correlated with the digestibility of the forage consumed. This relationship, allowing an alternate procedure for estimation of digestibility, was found to be expressed by the equation, $Y = 32.74 + 0.0168X + 8.47 \log X$, where: Y = per cent of dry matter digested, and X = units of chromogen per

gram of feces (dry basis). The standard error of the estimate of the digestibility of the consumed forage was 0.44%. The coefficient of correlation between digestion coefficients computed from this equation and those derived from the conventional digestion trial was 0.96 ± 0.01. Although it was not attempted, an equation of different form may fit these data slightly more satisfactorily than the one shown here.

When the quantity of dry matter excreted in the feces per unit of time is known, the intake of forage dry matter per unit of time may be computed by dividing the fecal dry matter by the per cent of the indigestible forage dry matter $(100 - Y)$.

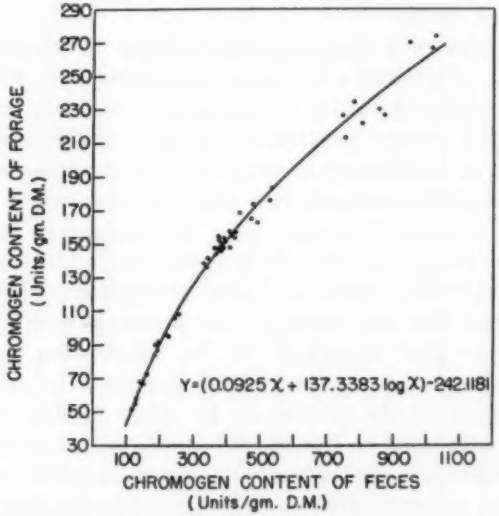

Fig. 1 Relationship of the chromogen concentration of feces to the computed chromogen concentration of the consumed forage.

In the following treatment of the data obtained in these studies, emphasis has been given to relating the chromogen concentration of the feces to the computed chromogen concentration of the consumed forage, from which relationship the digestibility of forage dry matter is determined. This scheme was adopted in order that a clearer presentation of the procedure could be made and in order that further applications of the findings could be considered.

The curvilinear relationship shown in figure 1 was found to exist between the chromogen concentration of the feces voided and the computed chromogen concentration of the

consumed forage. The equation expressing this relationship is:

(I) $Y = (0.0925\,X + 137.3 \log X) - 242.12$, where: $Y =$ units of chromogen per gram of forage (dry basis), and $X =$ units of chromogen per gram of feces (dry basis). The standard error of estimate of the chromogen concentration of the consumed forage was 0.55 units per gram. The coefficient of correlation between the computed chromogen concentrations of the forages and those predicted from equation I was 0.995 ± 0.001.

It will be observed that, regardless of the botanical composition, stage of growth and degree of digestibility within the ranges examined, the ratio of chromogen in feces to that in the consumed forage conformed to this relationship. The high degree of conformity as suggested by the small standard error of estimate demonstrates that the chromogen concentration of consumed forage may be accurately computed from a knowledge of the chromogen concentration in the feces. *In order that equation I may be applied satisfactorily, it is required that the value of the chromogen unit used be equivalent to that described in the calibration procedure above.* Arbitrary chromogen values assigned to extracts in the calibration of the procedure by other laboratories may be readily converted to the same chromogen values employed in these studies, either by comparing calibration equations (chromogen concentration — light transmission) or by employing the Na_2CrO_4 reference concentration.

The digestibility of forage dry matter, then, may be computed following actual measurement of the chromogen concentration of the feces, estimation of the chromogen concentration of the consumed forage from the above equation (I), and the fitting of these values into the following equation:

(II) Per cent digestibility of the dry matter $= 100 - \left(100 \frac{\text{units chromogen/gm forage, on dry basis}}{\text{units chromogen/gm feces, on dry basis}}\right)$.

Digestion coefficients predicted from the chromogen concentration in the feces are compared in table 2 with those

TABLE 2

A comparison of digestion coefficients estimated from the chromogen concentration of the feces with those derived from conventional digestion trials[1]

TRIAL NO.	DRY MATTER DIGESTIBILITY (%)							
	Forage 1		Forage 2		Forage 3		Forage 4	
	Conv.[2]	Est.[3]	Conv.	Est.	Conv.	Est.	Conv.	Est.
1	72.8 ± 0.70	73.7 ± 0.28	72.1 ± 1.16	71.3 ± 0.54	71.6 ± 0.91	71.1 ± 0.44	72.9 ± 0.29	75.1 ± 0.25
2	66.3 ± 0.20	65.5 ± 0.38	59.3 ± 0.34	60.0 ± 0.30	63.2 ± 0.24	62.7 ± 0.10	66.3 ± 0.78	68.1 ± 0.15
3	54.4 ± 0.08	53.7 ± 0.28	53.5 ± 0.88	53.0 ± 0.05	53.8 ± 1.23	53.8 ± 0.31	58.0 ± 1.67	55.5 ± 0.30
4	54.8 ± 0.38	54.6 ± 0.56	63.6 ± 1.19	64.1 ± 0.47	60.5 ± 0.82	61.7 ± 0.31	...	...
5A	...	...	...	...	60.2 ± 0.93	60.6 ± 0.47	...	...
5	...	...	61.8 ± 0.86	61.3 ± 0.41	60.1 ± 0.62	61.2 ± 0.22	...	...

[1] Digestion coefficients obtained with three steers in each case. Figures represent average digestion coefficients and standard error of the mean.

[2] Digestion coefficients obtained in conventional digestion trial.

[3] Digestion coefficients derived from concentration of chromogen in feces.

derived from conventional digestion trials. The coefficient of correlation between digestion coefficients determined conventionally and those computed from the fecal chromogen level was 0.985 ± 0.004. Although the digestibility of the dry matter of most pasture forages is within the range examined in these experiments, it is desirable that forages which are 80% or more digestible may eventually be studied.

The results of these studies demonstrate that this procedure circumvents the forage-sampling problem and allows an accurate estimate of forage digestibility under conditions simulating those of grazing. The relationship expressed by equation I, upon which this procedure of estimating digestibility

TABLE 3

Range of various forage characteristics examined

CHARACTERISTICS OF FORAGES	RANGE EXAMINED
Stage of plant growth	vegetative to seed
Ratio of leaves to stems[1]	7.5:1 to 1:2.1
Ratio of legume to non-legume forage	0:1 to 2.9:1
Average height of clipped forage (cm)	27.5 to 76.8
Crude protein content (% of dry matter)	6.79 to 22.05
Crude fiber content (% of dry matter)	26.75 to 37.25
Digestibility (% of dry matter)	51.6 to 74.0

[1] Heads are considered as stems in these data.

is based, was established as a result of feeding forages of varied physical, chemical and botanical composition. It, then, encompasses a wide variety of foraging conditions similar to those to which grazing animals are exposed. The range of the variations in some of the known characteristics of the forages examined in these experiments is shown in table 3.

Although it is clear that the digestibility of forages similar to those employed in these studies may be determined accurately, it is entirely possible that some plant species used as pasture herbage may not conform to the relationships described here. Also, it is possible that some of the plants composing the forage mixtures fed during the course of these

studies may not correspond if examined alone or as the predominant portion of a mixture. Pasture forages in which seeds compose a considerable portion of the dry matter, small grain crops and range browse employed as pasturage have not been examined to determine the extent to which they conform to these relationships. Studies of relatively pure stands of various common pasture forages are now in progress.

The data obtained in these experiments and in others (Woolfolk, '50) suggest that the plant chromogen employed as an indicator in these studies is related quantitatively in some way to those constituents which determine the digestibility of the plant as a whole. It is now well established that the digestibility of plants decreases as the stage of growth approaches maturity and that, accompanying this change, the proportion of the plant consisting of leaves decreases while the concentration of various plant constituents changes also. In these experiments it was observed that the chromogen level of forages decreased as the stage of growth approached maturity, as the ratio of leaves to stems decreased, and as the degree of digestibility decreased. The chromogen concentration of the leaves was found to be considerably greater than that of the stems.

Estimation of digestibility using small, randomly procured samples of feces

Although the estimated digestion coefficients shown in table 2 were derived from chromogen analyses conducted on 4-day composite samples of feces, it is conceivable that a duplicate set of chromogen analyses would not have represented more than a very few defecations during the 4-day fecal collection periods, owing to the small size (4 to 8 gm) of samples of fresh feces analyzed. In reality, therefore, these data may have been the result of analyses conducted on feces samples comparable to "grab" or "random fecal collection" samples.

It is indicated that, should a sample of feces obtained at any given time result in digestibility estimates equivalent to

those determined on feces samples compounded from total collection over a period of several days, a great advantage would be gained in terms of time and expense. Furthermore, this procedure would obviate the necessity of such impedimenta as harnesses and fecal collection bags.

In order to examine the feasibility of predicting digestibility from the analysis of small "grab" samples, a limited scale experiment was conducted in which samples weighing approximately 300 gm (50 gm, dry basis) were taken during each morning and evening of a 4-day period. The forage, which was clipped and hand-fed to three steers, was of the same source (theoretically the very same forage) as that being grazed by three similar animals. The results of this experiment are summarized in table 4. Although these data are too few to allow the validity of random sampling of feces to be established definitely, they do suggest that the degree of digestibility of the hand-fed forage and, therefore, the excretion of chromogen, were uniform as determined from samples of feces obtained in the morning and evening of 4 consecutive days. The digestion coefficients derived from "grab" samples agreed well with those estimated from the 4-day composite samples and those determined conventionally in hand-feeding trials. However, the digestibility of the forage selected by the grazing steers was not as uniform as that of the hand-fed grass, suggesting that a diet of variable composition was consumed by the grazing animals in this particular trial.

The grazing conditions imposed in this trial, in which the animals were restricted to small areas without opportunity for optimum selective feeding, probably resulted in less uniformity than would be observed under conditions of freer grazing. Despite these restricted grazing conditions, a striking degree of selectivity was manifested by the grazing animals. Based upon average data derived from three steers in each group, the hand-fed, clipped, whole forage was only 91.3% as digestible as the forage selected by the grazing ani-

mals. The results of a more extensive examination of selective feeding will be reported later.

Measuring forage consumption

It has been a major objective of the experiments reported here and others not yet reported to develop methods of meas-

TABLE 4

Digestion coefficients derived from the analysis of partial samples of feces obtained from steers hand-fed clipped, whole forage and grazing forage from the same source

DATE	SAMPLING TIME	HAND-FED STEERS			GRAZING STEERS		
		7	8	9	10	11	12
8/8	P.M.	61.9	62.1	63.0	70.3	67.6	69.5
8/9	A.M.	61.0	61.3	62.8	68.2	66.6	68.0
8/9	P.M.	61.6	61.9	62.2	67.2	66.2	68.7
8/10	A.M.	61.2	61.9	63.0	67.5	65.1	68.1
8/10	P.M.	61.8	62.4	62.6	67.7	66.2	68.7
8/11	A.M.	61.6	61.5	62.0	67.6	66.4	66.4
8/11	P.M.	60.8	61.8	62.3	68.7	63.8	68.9
8/12	A.M.	61.0	61.9	61.6	67.2	64.2	67.0
Ave. estimated coefficients based upon partial collection of feces		61.3 ± 0.15	61.8 ± 0.12	62.4 ± 0.18	68.1 ± 0.37	65.8 ± 0.45	68.2 ± 0.36
Estimated coefficients based upon 4-day composite sample of feces		61.4	61.7	62.8	68.3	66.1	67.9
Coefficient determined in conventional digestion trial		60.3	62.0	63.3	...	...	...

uring the rate of dry matter consumption as well as digestibility. An ideal method would appear to be one which allows accurate estimates of these measures to be made from small samples of feces taken at arbitrary times. Consequently, it is indicated that a combination of indicators, one naturally occurring in the forage (to be employed as an index of indigestibility) and another added to the animal's ration in known

quantity (to be used as an index of the total quantity of feces voided per unit of time), may accomplish, simultaneously, measurement of the digestibility and the rate of consumption of forages by grazing animals. Some data, which will be discussed in a later report, have been obtained from studies successfully employing chromium sesquioxide as an index of the total amount of feces voided per unit of time. If the use of chromium sesquioxide or of some other inert substance for the purpose of estimating the quantity of feces voided per unit of time should prove to be accurate, this procedure would circumvent the need for fecal collection bags, which presently are used on male animals but which are not very satisfactorily used on females. Since the combined data from the use of chromogen and chromium sesquioxide indicators are most promising in the estimation of the digestible dry matter intake of individual animals, it remains for experiments to be conducted which will ascertain an adequate fecal sampling procedure.

SUMMARY

The relationship between the chromogen-dry matter ratio of feces voided and that of forage actually consumed was established mathematically as a result of a study of 18 pasture forage mixtures ranging in dry matter digestibility from 51.6 to 74.0%. This relationship allowed the circumvention of the manual sampling of forage, as it was found that the chromogen concentration of the forage could be predicted from a knowledge of the fecal chromogen level. Data on the concentration of chromogen so estimated in the ingested forage may then be employed together with those on the chromogen concentration in the feces in the usual ratio technique for determining the digestibility of *consumed* forage under conditions of grazing. An alternate procedure for the direct computation of digestibility from the chromogen concentration of the feces was also suggested.

When the amount of dry matter voided in the feces per unit of time is known, the dry matter intake of grazing animals may be readily computed from a knowledge of the chromogen level of the *consumed* forage and of the feces, or from a knowledge of the degree of indigestibility of the forage.

Other applications of the principles of this method of measuring pasture forage digestibility and intake are considered.

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